THE PHYSIOLOGY OF DORMANCY AND GERMINATION IN CYSTS OF THE MARINE DINOFLAGELLATE SCRIPPSIELLA TROCHOIDEA

by

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for

Mom and Dad

Can we actually "know" the Universe?...

My God, it's hard enough finding your

way around in Chinatown.

-Woody Allen

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ABSTRACT

Cysts of Scrippsiella trochoidea are representative of the thick-walled non-motile resting cells produced by many dinoflagellates. Although the influence of cysts on the biology and ecology of dinoflagellates may be significant, little is known of the factors controlling cyst dormancy and germination, or of the metabolic foundations of these processes. This study addresses these issues using cysts produced and manipulated under defined conditions in laboratory culture.

Scrippsiella trochoidea cysts experienced a period of dormancy, lasting approximately 25 days, during which germination did not occur. The duration of this period was not affected by temperature. Cysts which had completed their dormancy period remained quiescent until permissive environmental conditions were established. Quiescent S. trochoidea cysts remained viable at 3°C in the dark for at least 350 days.

Germination in quiescent S. trochoidea cysts was photomorphogenically controlled: cysts deprived of light but otherwise provided with optimal environmental conditions failed to germinate. The light requirement was satisfied to a large extent by a single, brief, low intensity exposure (a photon fluence of 0.2 μmol m^{-2} "white" light elicited a 50% response). Yellow-green light ($\lambda \simeq$ 550nm) was found to be most effective.

Temperature exerted significant control over germination as well. Germination rate was maximal above 14°C; it decreased rapidly as temperature decreased below this level. Ultimate achieved germination frequency, in contrast, was relatively insensitive to temperature. The temperature range optimal for cyst germination did not precisely coincide with that for vegetative growth. Thus germination was greatly retarded at low temperatures which supported good vegetative growth, but on the other hand proceded optimally at high temperatures which completely inhibited such growth.

The most conspicuous compositional attribute of S. trochoidea cysts was their increased carbohydrate content, which was 10 times that of exponentially growing cells. Cysts contained significantly less protein and chlorophyll-a than vegetative cells, while the lipid

content of the two forms was comparable. The respiratory activity of quiescent cysts was estimated to be approximately 1.5% of that in vegetative cells. Although chlorophyll-a persisted in quiescent cysts, no photosynthetic activity could be detected therein.

The germination of S. trochoidea cysts was accompanied by an immediate increase in respiratory activity, with carbohydrate serving as the principal substrate. Protein synthesis became apparent within 24 hr of activation, followed by a dramatic increase in both chlorophyll-a and photosynthetic activity just prior to excystment.

Encystment and germination in S. trochoidea represent developmental patterns which are regulated by specific, albeit not yet well understood, environmental and biological parameters. The further elucidation of these processes and their regulation will lead to a better understanding of the dynamics of dinoflagellate populations in nature, as well as of the biology of dormancy and quiescence generally.

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INTRODUCTION

The widespread occurrence of dormant stages among a diverse group of organisms, from endospore-forming bacteria to hibernating mammals, attests to the fundamental importance of this phenomenon in biological systems. Some dormant forms are conspicuous in the life history and ecology of their species, and so have received much scientific attention. In this thesis I examine a dormant form which has been less well studied but which is nevertheless of potentially great biological and ecological significance: the dinoflagellate resting cyst. By examining dormancy and germination in cysts of dinoflagellates, I hope to add to our understanding of the ecology of these important members of the phytoplankton and to our knowledge of the phenomenon of dormancy in general.

In the introductory chapter that follows, I will briefly review the current state of knowledge concerning dinoflagellate cysts, other algal resting stages, and two of the more intensely studied resting systems - microbial spores and plant seeds. This discussion is followed by a summary of the results of the present work.

The data chapters of the thesis are presented as independent manuscripts. In Chapt. 1, some of the biological and environmental factors which control dormancy, quiescence, and germination in cysts of the study organism, Scrippsiella trochoidea, are examined. A special case of such control, and one which is unprecedented among dinoflagellates, is the photomorphogenic regulation of germination in these cysts. The relevant data are presented in Chapter 2. Finally, in Chapter 3, the gross biochemical and metabolic changes which

accompany encystment, dormancy, and germination in S. trochoidea cysts are addressed.

Additionally, two appendixes are included. In the first, evidence for the bacterial inhibition of encystment in S. trochoidea is presented. This topic is perhaps peripheral to the main concerns of the thesis, but is of sufficient interest to warrant its inclusion as an appendix. The second appendix contains methodoligical notes which supplement those presented in Chapters 1-3, and which should prove useful for the further pursuit of these studies.

BACKGROUND

Dinoflagellate Cysts.

A great many dinoflagellate species are known to include encysted stages in their life history. These cysts are generally thick-walled non-motile cells, morphologically distinct from their vegetative counterparts, and presumed to represent resting stages with extended longevity and increased resistance to adverse environmental conditions (Wall 1971, Dale 1983). While a few examples of asexual cyst production have been described, most dinoflagellate resting cysts are considered to be the product of sexual fusion (Loeblich and Loeblich 1984, Pfiester and Anderson 1986).

Although the occurrence of cysts among dinoflagellates has been known for over a century (see Loeblich and Loeblich 1984), their potential biological and ecological significance has only recently been widely appreciated (Wall and Dale 1968, Wall 1971, Anderson and Wall 1978). Among the roles that cysts have been suggested to play are survival through adverse environmental conditions, timing of bloom initiation and termination (through excystment and encystment, respectively), species dispersal, and genetic recombination (Wall 1971, Dale 1983, Anderson 1984).

Despite the potential importance of these roles in controlling the ecology and population dynamics of dinoflagellates, relatively little is known about cyst formation or germination. This lack of information arises in part from the difficulty of culturing dinoflagellates, and in part from the fact that most studies to date have been directed toward

the elucidation of dinoflagellate life histories, rather than their biological and environmental control. As culturing methods for dinoflagellates have improved, more studies involving the controlled manipulation of these organisms have been undertaken (e.g. Anderson et al. 1984). However, much of our information concerning dormancy and germination is still based on studies utilizing cysts recovered from the field.

The current state of knowledge regarding the biology of dinoflagellate cysts has been thoroughly summarized in two recent reviews (Dale 1983, Pfiester and Anderson 1986). The relevant studies are discussed in detail in Chapters 1-3 of this thesis as well. Therefore, only a brief review of dinoflagellate cyst biology will be presented here.

The common form of reproduction in dinoflagellates is asexual. In many species, sexual reproduction and subsequent cyst formation occur in response to nitrogen or phosphorus limitation (Pfiester and Anderson 1986). The ineffectiveness of light or temperature limitation in triggering sexual reproduction in some of these same species indicates that reduced growth rate alone is not responsible for the switch to sexuality (Anderson et al. 1984, Pfiester and Anderson 1986).

The fusion of two gametic cells results in a planozygote, which is often larger and more darkly pigmented than the vegetative cell. This zygote generally remains swimming for one to three weeks before becoming immotile and producing the heavy wall characteristic of resting cysts (Pfiester and Anderson 1986).

Once formed, most dinoflagellate cysts display a dormancy period, during which germination apparently cannot occur (Dale 1983, Pfiester and Anderson 1986). The definition of such dormancy periods has in many instances been based on casual observations of reduced germination success in young cysts under conditions conducive to germination in older cysts of the same species. Dormancy periods ranging from 12 hr to 6 mo have been reported in various dinoflagellate species (Huber and Nipkow 1923, Wall and Dale 1968, von Stosch 1973, Pfiester 1975, Anderson 1980, Endo and Nagata 1984). In some cases the duration of the dormancy period is affected by temperature: cold storage increases the dormancy period in Gonyaulax tamarensis, but decreases it in Peridinium cinctum (Dürr 1979, Anderson 1980).

The conditions necessary for germination in dinoflagellate cysts which have completed their dormancy period are not well defined. Most commonly, temperature has been considered the primary controlling factor, with low temperatures generally inhibiting germination and higher temperatures promoting it (Huber and Nipkow 1923, Wall et al. 1967, Wall and Dale 1968, 1969, von Stosch 1973, Pfiester 1975, Anderson and Morel 1979, Chapman et al. 1981, Endo and Nagata 1984, but see also Anderson 1980). The necessity for a temperature shift per se, as opposed to a simple requirement for incubation within a permissive temperature range, has not been clearly demonstrated to date (Anderson 1980, Dale 1983). Germination in the absence of any obvious environmental changes has been reported for a number of species (von Stosch 1973, Pfiester 1975, 1976, 1977, Pfiester and Skvarla 1979). In general, the effects on germination of environmental variables other

than temperature (eg. light, nutrient concentrations) have been discounted (Anderson and Wall 1978, Krupa 1981, Dale 1983).

The physiological bases of encystment, dormancy, and germination in dinoflagellates have not yet been directly addressed. However some information can be gleaned from light-microscope and ultrastructural studies. The widely reported inclusion of lipid globules and/or starch grains in cysts suggests that the production and accumulation of storage compounds accompanies cyst formation (Wall and Dale 1969, von Stosch 1973, Anderson 1980, Chapman et al. 1982). Studies in which the planozygote was observed indicate that this production occurs prior to the actual encystment of the cell (von Stosch 1973, Chapman et al. 1982).

The extensive ultrastructural rearrangement (compared to that of vegetative cells) reported in cysts of Woloszynskia tylota attests to the magnitude of metabolic changes which most likely accompany cyst formation (Bibby and Dodge 1972). In particular, the loss of membranous components from the cytoplasm, the aggragation of thylakoids and presence of lipid globules within the chloroplasts, and the condensed appearance of the chromosomes were taken by the authors to suggest a general reduction in metabolic activity. No direct measurements of metabolic activity in cysts have been reported to date.

In general, then, dinoflagellate cysts are assumed to represent resting stages which combine decreased metabolic activity with large accumulations of storage product to achieve increased longevity under conditions which preclude a normal photoautotrophic existence. While

nutrient limitation is most often responsible for the induction of sexuality and encystment, temperature is almost universally regarded as the environmental factor which controls germination.

In this thesis I have endeavored to rigorously examine some of these assumptions in cysts of Scrippsiella trochoidea, produced and manipulated under controlled laboratory conditions. In particular, I have addressed two general questions: 1) What biological and environmental factors affect dormancy and germination? and 2) What physiological changes accompany these phenomena? To the extent that I answer these questions in the following chapters, I will have provided a glimpse of the metabolic and molecular processes which underly dormancy and germination in dinoflagellate cysts.

The Organism: Scrippsiella trochoidea (Stein) Loeblich (syn. Peridinium trochoideum (Stein) Lemmerman).

Scrippsiella trochoidea is a small thecate photosynthetic dinoflagellate of wide neritic distribution. It was chosen for the present study primarily because it grows, encysts, and germinates readily in laboratory culture. The relatively short dormancy period of its cysts, and the ease with which their germination can be assessed (Chapt. 1), further contributes to the suitability of S. trochoidea for laboratory study.

Some confusion currently surrounds the taxonomy of Scrippsiella-like dinoflagellates (Balech 1974, Dale 1977, Dodge 1982). I based the identification of the clone used in this study on the number, shape, size, and position of the thecal plates (K.A. Steidinger, pers. comm.),

in combination with the presence of a calcareous cyst wall (Wall and Dale 1968, Dale 1977).

The cysts of S. trochoidea have been described by a number of workers (Braarud 1958, Wall and Dale 1968, Wall et al. 1970, Watanabe et al. 1982, Anderson et al. 1985). Cysts are ellipsoidal, approximately 28 μm in length and 23 μm in width (as measured in the present study). The characteristic spiny cyst wall is composed of calcite (Wall et al. 1970). It is formed within the zygotic theca and is reportedly surrounded by an outer membrane, at least in freshly formed cysts. Calcareous cyst walls are relatively uncommon among dinoflagellates, occurring only in members of Scrippsiella and a closely related marine genus, Ensiculifera (Dale 1977, 1983). However, in possessing an acid-resistant inner wall (Wall et al. 1970), and one or two orange/red "stigmata," S. trochoidea cysts are similar to the cysts of many other dinoflagellate species (Dale 1983).

Upon germination, the cyst wall splits open, usually latitudinally, and a single motile cell emerges. Meiosis presumably occurs within the diploid germling some time later to reinitiate haplontic vegetative growth.

Dormancy: Concepts and Terminology.

The diverse manifestations of "dormancy" throughout the biota makes adoption of a universal vocabulary for dormancy-related phenomena difficult and potentially misleading. Thus, the definitions put forth by Sussman and Halvorson (1966) are useful in considering the behavior of microbial spores, but are less so in considering plant seeds, for

which a separate vocabulary has been adopted (Nikolaeva 1977, Bewley and Black 1982).

In this thesis I employ the latter group of definitions, as suggested by Pfiester and Anderson (1986), and as discussed more fully in Chapt. 1. The general ease with which the phenomena observed in the present study are described within the context of these definitions argues strongly for there use here. "Dormancy," then, will refer to the state in which a viable cyst fails to germinate, regardless of environmental conditions. Such a cyst is presumed to be inhibited from germination by some endogenous factor. On the other hand, cysts which are competent to germinate but which are prevented from doing so by unfavorable environmental conditions will be referred to as "quiescent." This dichotomy of resting states, one resulting from a property of the cell itself and the other from a property of the environment, is an important concept, and is present (though differently named) in both the vocabularies referred to above. The manifestation of this dichotomy in the case of S. trochoidea cysts will become apparent (Chapt. 1).

The definition of dormancy I have adopted is purely operational. So defined, dormancy may result from the need for further development or from a specific metabolic block. In the former case, a general reduction in metabolic activity would not necessarily be associated with the dormancy period. Seed biologists refer to these two types of dormancy as "morphological" and "physiological" dormancy, respectively. In the case of a single-celled organism, the distinction between "development" and metabolic change can sometimes be tenuous.

However I feel that the conceptual difference is still valid, and will occasionally refer to the distinction between these two general dormancy types in the discussions that follow.

Finally, for general discussions about dormancy-related phenomena in a range of organisms, I will use the term "rest" to refer to any "reversible interuption of the phenotypic development of an organism" (after Sussman and Halvorson's [1966] definition of dormancy). I therefore intend "rest" to encompass both dormancy and quiescence. The common definition of "rest" notwithstanding, no a priori assumption of a reduction in metabolic activity is implied.

Resting Stages in Other Algae.

Resting cells of some sort are produced by members of most algal classes (Erben 1962, Fryxell 1983). Examples of both sexual resting stages (eg. hypnozygotes) and asexual resting stages (eg. akinetes, hypnospores, resting spores) are common. In many cases a thickened cell wall is the single criterium used to characterize an algal life history stage as a "resting" stage (Coleman 1983).

As is the case for dinoflagellate cysts (see above), nitrogen depletion is the factor most often cited as inducing the production of both diatom resting spores (Hargraves and French 1983) and green algal hypnospores, akinetes, and hypnozygotes (Coleman 1983). There is little data concerning the environmental control of chrysophyte statospore production, but again, N limitation appears to promote encystment under certain conditions (Sandgren 1981). Although the data for cyanophyte akinete production is varied and in some cases

contradictory (Carr 1979), light limitation (rather than nutrient limitation) has been recently implicated as a general controlling factor (Sutherland et al. 1979). Nitrogen and phosphorus limitation have been shown to inhibit akinete formation in at least one cyanophyte (Sutherland et al. 1979).

The presence of a dormancy period among algal resting stages is not commonly noted, so that if dormancy occurs it probably lasts on the order of days in most cases, rather than weeks to months as observed in many dinoflagellates. A conspicuous exception to this generalization is the resting spore of the pennate diatom Eunotia soleirolii, which requires a dark (preferably cold) pretreatment of 4 weeks or more before germination can occur (von Stosch and Fecher 1979). Hargraves and French (1983) note that many centric diatoms display a short "refractory period," lasting days to weeks, during which germination does not take place. However these authors distinguish this phenomenon from the dormancy period in E. soleirolii on the basis of the apparent lack of specific environmental requirements during this period. Although mature akinetes of the cyanophyte Anabaena cylindrica are reported to germinate without delay (Carr 1979), Aphanizomenon flos-aquae akinetes recovered from the field apparently experience a dormancy period lasting at least 5 months (Wildman et al. 1975).

Restoration of standard culturing conditions, in terms of nutrients, light, and temperature, is in all cases sufficient to permit germination in non-dormant algal resting cells. However, the extent to which each of these factors specifically exerts control over germination is in most instances not clear. Dark, cold conditions

appear to prevent germination (ie. to maintain quiescence) in nearly all forms (Spencer et al. 1980, Hollibaugh et al. 1981, Rai and Pandey 1981; but see also von Stosch and Fecher 1979). Light is generally necessary for germination in diatom resting spores (Hollibaugh et al. 1981, Hargraves and French 1983; but again, see von Stosch and Fecher 1979) and in cyanophyte akinetes (Yamamoto 1976, Braune 1979, Chauvat et al. 1982), but is apparently unnecessary in chlorophyte akinetes (Neal and Herndon 1968).

Note that the requirement for light in both diatoms and cyanophytes is satisfied only by relatively high photon fluences, and is therefore fundamentally different from the light response I will report on in S. trochoidea (Chapt. 2). The extent to which this high threshold response involves photosynthesis has not been addressed in diatom resting spores, and remains controversial in cyanophyte akinetes (Chauvat et al. 1982, Braune 1979).

Nutrients exert a strong influence on diatom resting spores, many species of which are prevented from germinating under otherwise optimal conditions by nitrogen starvation (Hargraves and French 1983; but see also Hollibaugh et al. 1981). In contrast, many cyanophyte akinetes germinate in the same medium in which they were formed, in the absence of added nitrate or phosphate (Singh and Sunita 1974, Wildman et al. 1975, Yamamoto 1976; but see also Rai and Pandey 1981).

The physiology of quiescence and germination in these various algal stages is discussed in detail in Chapt. 3. The most obvious characteristic common to all of these forms is the accumulation of storage products: cyanophycin and glycogen in cyanophyte akinetes

(Wildman et al. 1975, Sutherland et al. 1979), lipid and/or starch in the others (Anderson 1975, Berkaloff and Kadar 1975, Lichtlé 1979, Doucette and Fryxell 1983, O'Neal and Lembi 1983).

Generalities concerning the metabolic activity of algal resting stages are difficult to make. Examples of reduced resting stage respiration (relative to the vegetative state) are found among cyanophytes and diatom resting spores (Anderson 1976, French and Hargraves 1980, Chauvat et al. 1982), but increased or comparable respiration has been reported in akinetes of other cyanophytes (Fay 1969a, Yamamoto 1976) and in those of chlorophytes (O'Neal and Lembi 1983). Likewise, although reduced photosynthetic capacity is found in cyanophyte and chlorophyte akinetes (Fay 1969a, Yamamoto 1976, Chauvat et al. 1982, O'Neal and Lembi 1983), photosynthetic activity comparable to that in vegetative stages has been recorded in many diatom resting spores (French and Hargraves 1980, Hollibaugh et al. 1981).

The metabolic changes accompanying germination generally involve a restoration of activity approaching vegetative levels, although the details of these changes vary (see Chapt. 3). The developmental program suggested by Chauvat et al. (1982) for cyanophyte akinete germination provides a convenient framework for considering such metabolic changes. Thus, increased respiration of endogenous reserves precedes (and is presumably a prerequiste for) increased RNA and protein synthesis, which in turn contribute to the reconstitution of the photosynthetic system and the ultimate return to photoautotrophic metabolism. This paradigm is consistent with the metabolic and compositional data for germination in chlorophyte akinetes (O'Neal and

Lembi 1983) and in S. trochoidea cysts (Chapt. 3). The molecular mechanisms by which these metabolic changes are regulated in germinating algal resting cells are unknown at present.

The Resting State and Germination in Other Systems.

In light of the limited available data regarding the physiology of dinoflagellate cysts specifically, and algal resting stages in general, it may be informative to consider the state of knowledge concerning the resting state and germination in better studied biological systems. Two resting forms for which a wealth of information is available are bacterial endospores and plant seeds. Rather than attempt to synthesize the truly staggering volume of data concerning each of these systems, I will consider these two examples in the narrow context of the metabolism and molecular regulation of dormancy, quiescence, and germination. For further information regarding these resting forms the reader is referred to the many excellent reviews currently available (bacterial spores: Sussman and Halvorson 1966, Gould 1970, 1977, Warth 1978, Hanson 1979; plant seeds: Khan 1977, Taylorson and Hendricks 1977, Mayer and Marbach 1981, Bewley and Black 1978, 1982).

Respiratory activity in resting stages is generally much lower than in vegetative forms. In microbial spores, respiration rates are reduced by factors of 20 to 100, relative to that in vegetative cells (Sussman 1966, Dresser and Broda 1969, Sussman 1969). Note that these reductions, which are comparable to those I report for S. trochoidea cysts (Chapt. 3), are for hydrated spores. Dessication of these same spores results in far greater respiratory reductions without a loss in

viability (Dresser and Broda 1969). Likewise, respiratory activity in "dry" plant seeds is greatly reduced compared to that of imbibed (but still dormant) seeds (Bewley and Black 1979, 1982). Although increases in respiration generally accompany germination, the role of such increases as primary events in the termination of dormancy and quiescence has not been demonstrated (see below).

Bacterial spores usually require specific "activation" treatments before germination can procede. Sublethal heating, ageing, low pH, strong oxidizing agents, and compounds containing sulphydryl groups are generally effective activators (Keynan and Evenchik 1969; Hanson 1979). The critical change caused by these treatments is unknown, but often increased respiration rate and enzymatic activity are observed. In the absence of subsequent germination, these changes may gradually reverse (Keynan and Evenchik 1969). After activation, exposure to an appropriate germinant results in germination of the spore. Examples of germinants include many nutritional substances (L-alanine is commonly effective), non-nutritional compounds (e.g. Ca-dipicolinate), and enzymes (e.g. lytic enzymes). Physical treatments such as abrasion and pressure often promote germination as well (Gould 1969; Gould and Dring 1972).

Within a minute or two of the application of a germinant, heat resistance is lost, the peptidoglycan of the spore cortex is hydrolysed, Ca-dipicolinate is released, and the protoplast swells and becomes metabolically active (Gould and Dring 1972; Hanson 1979). ATP concentration increases 100 fold over the first 5 minutes, apparently at the expense of 3-phosphoglycerate which is present in high

concentrations in the dormant spore (Setlow and Kornberg 1970a). RNA and protein synthesis become detectable in the first few minutes after germination (Setlow and Kornberg 1970b).

The events primarily responsible for activation and germination do not appear to involve macromolecular synthesis. The insensitivity of the early stages of germination to inhibitors of RNA and protein synthesis supports this hypothesis (Keynan and Halvorson 1965; Sussman and Halvorson 1966). Further support is gained from studies on the in vitro activity of components extracted from dormant spores. Although some TCA cycle enzymes and many biosynthetic enzymes cannot be detected in such preparations, the enzymes necessary for the metabolism of 3-PGA to acetate and CO_2 are present, and their activity is sufficient to account for the ATP production evident during early germination (Setlow and Kornberg 1970a, Hanson 1979). Furthermore, spores contain all or nearly all the components necessary for protein synthesis, although no such synthesis is detectable until after germination (Kobayashi et al. 1965; Deutscher et al. 1968; Sussman and Douthit 1973).

Deficiencies in the metabolic and synthetic machinery of spores, then, seem insufficient to account for the regulation of activity in the dormant state. Instead, the activity of these components is somehow repressed in spores and "unmasked" during activation and germination. The mechanisms by which such changes occur remain unknown. Hypothetical mechanisms include self inhibitors, spatial partitioning of components, reversible binding of key molecules, tertiary structural changes, and alterations in the intracellular ionic

environment (Keynan and Evenchik 1969; Gould 1969; Gould and Dring 1972, 1975; Sussman and Douthit 1973).

A diversity of resting behaviors is found among plant seeds (Nikolaeva 1977). Dormancy occurs in many seeds, although in domesticated species it has generally been shortened or eliminated. The mechanisms by which dormancy is maintained in seeds, and conversely, the mechanisms by which specific treatments end dormancy, are not known (Bewley and Black 1982). In some cases (termed "morphological" dormancy), embryos are obviously underdeveloped and the breaking of dormancy is simply a matter of waiting for development to be completed (Nikolaeva 1977). In others (termed "exogenous" dormancy), seed coat effects (eg. reduced permeability to oxygen or water, or mechanical resistance to embryo expansion) appear responsible. However, a large group of seeds display "physiological" dormancy. In this case, morphologically fully developed embryos, excised from their seed coats, fail to germinate in the absence of a specific activating treatment. Such treatments commonly include cold moist storage ("stratification"), dry storage ("afterripening"), light exposure, specific temperature regimes, and various combinations of these (Nikolaeva 1977, Bewley and Black 1982).

The mechanisms by which these treatments terminate dormancy is unknown. A host of physiological and metabolic changes have been demonstrated to occur in response to such treatments, but a specific role for any of these changes in breaking dormancy has yet to be convincingly demonstrated (Bewley and Black 1982). In the case of dormancy termination by exposure to red light, the primary triggering

event is almost certainly the photoconversion of phytochrome from the P_r to the P_{fr} form (Bewley and Black 1982). However, the mechanisms by which this primary event is translated to a release from dormancy remains unknown. In the case of other dormancy-breaking treatments, identification of even the primary event has proven to be very difficult.

The observation that germination in dormant seeds is often stimulated by elevated O_2 tensions and, paradoxically, by various respiratory inhibitors, has led to the hypothesis that activation involves an oxidative process (Roberts 1969, Roberts and Smith 1977). Increased O_2 levels would directly favor this process, while inhibitors would supposedly make O_2 available by depressing competing oxidative pathways. Roberts (1969) suggested that the pentose phophate pathway is the oxidative process important in breaking dormancy. While some evidence indicates that the activity of this pathway is higher in activated seeds than in constitutively dormant seeds, the overall importance of the pathway in the breaking of dormancy, and the mechanisms by which it is suppressed before activation, are unknown (Bewley and Black 1978, Bewley 1979, Bewley and Black 1982).

Hormones have also been suggested to play a major role in the regulation of dormancy in seeds (Wareing and Saunders 1971; Khan 1975; Taylorson and Hendricks 1977; Bewley 1979, Bewley and Black 1982). Gibberillic acids are effective stimulants for germination in a wide variety of dormant seeds, while abscisic acid often acts as an inhibitor of germination. The role of these and other hormones in the natural regulation of dormancy has not been easy to establish. The

general lack of correlation between the levels of particular hormones and degree of dormancy has led to the hypothesis that the balance between different hormones, rather than the levels themselves, regulate dormancy and germination (Khan 1975; Taylorson and Hendricks 1977). While changes in endogenous hormonal levels are often observed during activation, it is not known whether these changes result in, or are the result of, such activation. At present, the molecular mechanisms for the regulation of dormancy by hormones is not understood (Bewley 1979). Furthermore, the mechanisms by which activation treatments such as stratification might influence the levels of hormones in seeds are unknown.

Imbibation is the prerequisite for germination in non-dormant seeds. Within minutes of the initial contact with water, respiration rate rises sharply, ATP content increases, and protein and RNA synthesis commence (Taylorson and Hendricks 1977; Bewley and Black 1978; Delseny et al. 1980/81). The regulation of these early events is not understood at present. Dry seeds do respire, although there is evidence that their respiratory pathways are impaired (Bewley and Black 1978). The presence of mRNA, as well as all the other components necessary for protein synthesis, has also been demonstrated in many dry seeds.

These observations suggest that macromolecular synthesis is not a prerequisite for the early germination events in seeds, although direct evidence for this hypothesis is scarce at present. While rehydration per se is probably important in stimulating mitochondrial and synthetic activity, its role as the primary effector of germination has not been

established. Other mechanisms such as the outward diffusion of endogenous repressors, or the activation of derepressors, may be involved (Mayer and Shain 1974; Bewley and Black 1978).

In both microbial spores and plant seeds, then, dormancy and quiescence appear not to be the product of grossly disrupted metabolic or synthetic pathways, but rather to result from the repression of otherwise functional cellular machinery. Despite intense study, the mechanisms of such repression, and the events directly involved in the termination of this repression during activation, remain elusive.

SUMMARY OF RESULTS

A detailed knowledge of the dormancy and germination behavior of Scrippsiella trochoidea cysts is necessary before the physiological bases (or, for that matter, the ecological consequences) of these phenomena can be considered. Such knowledge is developed in Chapts. 1 and 2 of this thesis. Two questions of primary importance can be addressed in this regard: 1) Is there a dormancy period in S. trochoidea cysts? and 2) Once any such dormancy is broken, what are the conditions necessary and sufficient for cyst germination?

The data presented in Chapt. 1 indicate that S. trochoidea cysts experience a dormancy period lasting approximately 25 days. Cysts younger than this fail to germinate under conditions which support germination in older cysts. The duration of dormancy is insensitive to temperature; cysts stored at 3°C or 18°C display identical minimum germination ages. The extent to which the dormancy period in S. trichoidea reflects the gross development of an immature cyst rather than a period of endogenous metabolic and developmental repression of an otherwise competent cell cannot be discerned at present (but see Chapt. 3 discussion).

Temperature has traditionally been considered the environmental factor which exerts primary control over germination in non-dormant dinoflagellate cysts. Although I find for S. trochoidea cysts that light might best be assigned the role of "primary" regulator (Chapt. 2), the data in Chapt. 1 demonstrate that temperature does exert significant influence on germination as well. Optimum germination rate

is achieved between approximately 14°C and (at least) 25°C. Below 12°C germination rate decreases sharply; median germination time at 3°C was greater than 75 days (compared to 3- 5 days at optimal temperatures). Despite the marked decrease in germination rate at sub-optimal temperatures, the ultimate germination frequency achieved appears insensitive to temperature, at least for temperatures above 5°C. Comparable frequencies might have been observed at temperatures below 5°C had the length of the experiment been sufficient. Thus, the possibility of very slow germination at temperatures below 3°C cannot be discounted.

The role of nutrients in controlling germination is problematic. Scrippsiella trochoidea cysts did germinate in Sargasso seawater and in the N-depleted medium in which they were formed. However, the rate of germination under these conditions was generally slower than that in nutrient replete medium. Therefore, nutrients appear to facilitate germination, but they are not absolutely required. The high rate of germination in nutrient-poor medium among cysts pre-treated under cold, dark conditions suggests that the response to nutrients by cysts can be modulated by age or storage treatment.

Scrippsiella trochoidea cysts require light for germination. This conclusion is by far the most surprising result of this thesis. Only two other studies have shown any effect of darkness on dinoflagellate cyst germination, and in both of these cases germination was retarded, but not prevented (Endo and Nagata 1984, Anderson unpubl.). Although my initial experiments yielded similar results (Chapt. 1), when extraordinary precautions were taken to avoid inadvertent light

exposure during cyst storage and manipulation, germination did not occur (Chapt. 2). Exposure to light of these same dark-stored cysts resulted in rapid germination.

The requirement for light by S. trochoidea cysts is to a large extent satisfied by very short (1 s or less) low intensity exposures. Significant germination was observed in response to photon fluences (= light exposure, integrated over time) as low as 0.04 μmol m^{-2} "white" light. Considering that the photon fluence rate can be integrated over at least 1000 s by S. trochoidea cysts, a germination response would be expected after a 15 min exposure to white light at an intensity of 4 x10^{-5} μmol m^{-2} s^{-1}. This fluence rate is on the order of 10^{-7} - 10^{-8} times full sunlight! Cysts are most responsive to yellow-green light ($\lambda \simeq$ 550nm), with reduced germination occurring in response to lower (blue) wavelengths, and no germination detected at higher (red) wavelengths. The ecological relevence of these light intensities and spectral characteristics is discussed in Chapt. 2.

The very low photon fluence requirement for light-triggered germination, combined with its relative insensitivity to blue and red light, argues against the involvement of photosynthesis in this response. The apparent inability of cysts to photosynthesize immediately subsequent to activation (Chapt. 3) lends further support to this conclusion. However, it is important to note that in no treatment involving single short light exposures was germination frequency quite as high as that achieved under continual 14:10 hr daily exposure. Therefore, a photosynthetic role in promoting germination

cannot be totally excluded. It is possible that the stimulatory effect of nutrients on germination, noted above, could be mediated through such photosynthetic promotion.

The changes in composition and metabolic activity which accompany encystment and germination in S. trochoidea are addressed in Chapt. 3. The major compositional difference between vegetative cells and cysts is the dramatic accumulation of carbohydrate in the latter. Protein and chlorophyll-a content is significantly reduced in cysts relative to exponentially growing cells, while lipid content of the two stages is comparable.

Reduced respiratory activity is clearly correlated with dormancy and quiescence in S. trochoidea, with oxygen consumption in cysts estimated at between 1.5% to 10% that of vegetative cells. Respiration in dormant cysts appears higher than in quiescent cysts, based on the loss of carbohydrate and lipid, although it is still an order of magnitude below the vegetative rate. The anomalous effect of temperature on respiration rate in quiescent cysts, and the apparent net increase in lipid in these cysts, are two noteworthy metabolic features which require additional attention.

The transfer of quiescent cysts to permissive conditions results in an immediate (within 12 hr) increase in respiration rate, as evidenced both by carbohydrate loss and by O_2 consumption. This increase in respiratory activity is followed by an increase in protein content and, later, by an increase in chlorophyll-a and an elevation of photosynthetic capacity. Just prior to germination, the P/R ratio becomes greater than 1, and the estimated per-chlorophyll

photosynthetic activity is 75% that of vegetative cells. Complete restoration of photosynthetic and respiratory activity apparently is not achieved until after emergence of the planomeiocyte and the reestablishment of a planktonic existence.

Conclusion.

Scrippsiella trochoidea cysts represent true "resting" cells, containing extensive carbohydrate reserves and displaying greatly reduced metabolic activity. After completing their dormancy period, which lasts approximately 25 days, cysts remain quiescent until environmental conditions suitable for germination are established. Quiescent S. trochoidea cysts remain viable for at least a year under cold, dark conditions.

Despite their low level of activity, quiescent cysts remain poised to germinate and reestablish a planktonic autotrophic existence within days of the onset of permissive conditions. Under such conditions a rapid and dramatic increase in respiratory activity occurs, followed by protein synthesis and, just prior to excystment, by chlorophyll-a synthesis and reactivation of the photosynthetic system.

Germination in quiescent S. trochoidea cysts is influenced by light, temperature, and nutrient conditions. The photomorphogenic control of germination, as observed here, is unprecedented among dinoflagellate studies. The low threshold of the response, combined with the relative effectiveness of the green wavelengths, argues strongly that this control is not exerted through photosynthesis, but is rather mediated by a specific photoreceptor system. While this

system appears primarily responsible for turning germination "on" and "off," the rate at which such germination procedes can be significantly affected by nutrient concentrations and temperature. The influence of temperature, in particular, is very strong: germination rate at 6°C is an order of magnitude below the optimal rate (at 14°C and above), although vegetative growth rate is still 70% of the maximum at this temperature. In contrast, at temperatures above 22°C germination procedes optimally, while vegetative growth is largely inhibited.

The ability of S. trochoidea cysts to germinate under nutrient, light, and temperature conditions which are not conducive to vegetative growth demonstrates that these two processes are regulated independently. Germination is not simply the result of the stimulation of metabolic functions through the restoration of optimal growth conditions. In like manner, encystment and quiescence do not result simply from the onset of conditions sub-optimal for growth. Rather, both encystment and germination represent distinct developmental patterns which are regulated by specific, albeit not yet well understood, environmental and biological parameters. The further elucidation of the regulation of these processes will undoubtedly lead to a better understanding of the dynamics of dinoflagellate populations in nature, as well as of the biology of dormancy, quiescence, and germination in biological systems generally.

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Chapter 1

Biological and Environmental Control of Germination in *Scrippsiella trochoidea* Cysts

INTRODUCTION

A great number of dinoflagellates produce resting cysts during their life history (Wall 1971, Dale 1983). Many roles have been suggested for these cysts (and for algal resting stages in general), including short- and long-term survival of unfavorable conditions, bloom initiation, species dispersal, reproduction, and preservation of genetic variation (Wall 1971, Anderson and Wall 1978, Coleman 1983, Dale 1983, Anderson 1984). The importance of each of these roles to dinoflagellates in nature has yet to be established. The extent to which a cyst could fulfill any particular function will depend, in part, upon the physiology and environmental regulation of its dormancy and germination. Thus, knowledge of these processes would lead to a better understanding of the roles played by cysts in nature, and of the ecology of dinoflagellates in general.

The data concerning dormancy and germination in dinoflagellate cysts is rather limited (see reviews by Dale 1983, Pfiester and Anderson 1986). Most relevant studies have had as their major goal the elucidation of dinoflagellate life histories, rather than the investigation of the physiology underlying the observed transformations.

The most detailed study to date dealing with this latter concern was published 60 years ago by Huber and Nipkow (1923). These authors examined the effects of various environmental parameters on the germination of cysts of Ceratium hirundinella recovered from lake sediments. They found that temperature and cyst age exerted a

pronounced influence on germination, while nutrients and light appeared less important. Germination was inhibited in one and a half month old cysts, proceded suboptimally in three month old cysts, and was excellent in seven and a half month old cysts, suggesting a period of dormancy during which germination is retarded. Germination rate in C. hirundinella was maximum at 21-24°C, and dropped at temperatures above or below this range. Cysts germinated in distilled water and in darkness, indicating that neither external nutrients nor light was required.

More recent studies have extended these results to other dinoflagellate species, both freshwater and marine, but the generalities which first arose from Huber and Nipkow's work have remained largely unaltered. Thus, "dormancy periods" lasting on the order of weeks to months have been noted in a number of species (Wall and Dale 1968, von Stosch 1973, Pfiester 1975, Anderson 1980, Endo and Nagata 1984). These reports have, for the most part, consisted of casual observations of reduced germination in young cysts under conditions which are conducive to germination later on. Anderson (1980) presented more detailed data for Gonyaulax tamarensis cysts from the field, demonstrating a gradual increase in germination success as these cysts aged.

The importance of temperature in regulating germination, as reported by Huber and Nipkow (1923), has likewise been confirmed. In general, low temperatures inhibit germination, while shifts upward promote it (Wall et al. 1967, Wall and Dale 1968, 1969, von Stosch 1973, Pfiester 1975, Anderson and Morel 1979, Chapman et al. 1981, Endo

and Nagata 1984). Gonyaulax tamarensis germinates in response to a downshift in temperature (22° to 15°C) as well as to the traditional upshift (4° to 15°C) (Anderson 1980).

In addition to directly controlling germination rate, temperature may indirectly affect germination by influencing cyst dormancy. Thus, while lower temperatures generally prevent germination, cold pretreatments apparently shorten the mandatory dormancy period in Peridinium cinctum (Dürr 1979), and result in better synchronized and more complete germination in Gymnodinium pseudopalustre and Woloszynskia apiculata upon restoration of permissive temperatures (von Stosch 1973). On the other hand, cold storage has been shown to lengthen the dormancy period in G. tamarensis and in a Peridinium sp. (Anderson 1980, Endo and Nagata 1984).

Little data concerning the effects of other environmental parameters, such as light or nutrient concentrations, are available. It is generally held that these factors do not exert a strong influence on excystment, though they obviously would greatly affect subsequent survival and growth of the germling (Anderson and Wall 1978, Dale 1983).

There is a clear need for more detailed data regarding the dormancy and germination behavior of dinoflagellate cysts. In particular, controlled studies involving cysts produced, stored, and manipulated under defined conditions are lacking. This chapter reports the results of such a study using culture-produced cysts of the marine dinoflagellate Scrippsiella trochoidea. This species is a common component of the neritic phytoplankton community worldwide, and it grows and encysts relatively well under laboratory conditions (Braarud 1958, Wall et al. 1970, Watanabe et al. 1982).

METHODS

The Organism. Initial experiments employed a non-axenic multi-clonal culture of Scrippsiella trochoidea, designated ScrpMxB, originally isolated from Perch Pond (Falmouth, MA) by D. M. Anderson. A clonal axenic culture (designated SA10) was subsequently established from ScrpMxB by repeatedly rinsing individually isolated motile cells in drops of sterile medium. SA10 was routinely tested for contamination using various marine bacteria growth media (Hoshaw and Rosowski 1973); occasional direct inspection of old cultures, under phase contrast illumination at 400x, confirmed the absence of bacterial contaminants. Species identification of this clone was confirmed by K. A. Steidinger (Pers. Comm.).

Culturing. General procedures were as outlined in Anderson et al. (1984). Scrippsiella trochoidea cultures were routinely grown in f/2-enriched Vineyard Sound seawater (31 ppt), minus silicate, in 25x150mm borosilicate culture tubes (Guillard and Ryther 1962). For cyst production, cultures were grown in reduced-nutrient media. The standard encystment medium was 1/10x f/2-enriched seawater, with 5μM ammonium replacing nitrate as the source of nitrogen. Media was autoclaved complete with nutrients; in contrast to the case for Gonyaulax tamarensis (Anderson et al. 1984), growth and encystment in S. trochoidea were not adversely affected by media thus prepared. Cultures were routinely maintained at 18°C under a 14:10 hr daily light-dark cycle, with cool white fluorescent bulbs providing illumination at approximately 450 $\mu E\ m^{-2}\ s^{-1}$ PAR.

Experiments addressing the effects of temperature on growth rate and germination were performed using a temperature gradient bar, as described in Anderson et al. (1984). Growth rate was calculated from in vivo fluorescence (Turner Designs Model 10) of well acclimated cultures considered to be in balanced growth (Brand et al. 1981).

Cyst storage and germination. After cysts appeared in culture, the tubes were generally stored without further manipulation. Since storage and germination conditions were varied experimentally, each particular treatment employed will be noted in the Results section. For the sake of discussion (and for want of a better term), I will refer to the start of experimental germination treatments as "activation." This definition is completely operational, and should not be confused with the physiological process of activation often discussed in connection with bacterial spores.

Germination experiments were conducted in two ways. In early experiments, individual cysts were isolated by micropipette into 130 µl of medium in the wells of 96-well tissue culture plates (Costar, Cambridge, MA). These plates were then placed under the specified germination conditions and inspected periodically at 100x on an inverted microscope. The appearance of swimming S. trochoidea cells indicated that germination had occurred. At least 50 cysts were isolated and scored for each experimental treatment.

During the course of these initial experiments, it became clear that the calcitic cyst wall which was left after germination remained intact and was easily recognized. Thus the appearance of empty cysts, rather than swimming cells, could be used as an indicator of

germination. Cyst suspensions, instead of individually isolated cysts, could therefore be employed in experiments. Periodically scoring the proportion of empty cysts in culture (usually 0.1 ml aliquots in a Palmer-Maloney slide) is far more convenient, and allows greater experimental flexibility, than searching for the appearance of swimming cells; this method was used exclusively in subsequent experiments.

For some experiments, cysts were separated from other cells and resuspended in the specified medium for storage or germination. Following concentration by centrifugation (typically 500 xg for 20 min in a horizontal rotor), cysts and vegetative cells were separated using a Percoll-sorbitol density step-gradient. "Percoll-SSW" (Price et al. 1978) without tris was employed. Solutions of Percoll + sorbitol and $MgCl_2$ + seawater were autoclaved separately, and combined aseptically just prior to use; pH of the combined solution was 7.9- 8.1, its density was approximately 1.15 g ml^{-1} (Price et al. 1978). After centrifugation as above, cysts formed a pellet at the bottom of the tube, while vegetative cells accumulated at the Percoll-seawater interface.

RESULTS

Encystment. In encystment-medium batch cultures, S. trochoidea grows exponentially at a rate of approximately 1 doubling per day (Fig. 1). A day or two after stationary phase is reached, the first cysts appear; cyst abundance then increases over the next 3 or 4 days. Final cyst yield is typically 10% of the maximum vegetative cell number.

Age Effects. The pattern of germination for cysts of different ages is shown in Fig. 2. These cysts were produced and stored at 18°C under a 14:10 hr L:D cycle. After 4, 13, 20, and 29 days, cysts were isolated from culture and incubated at 15°C in f/2 medium (light regime unchanged). A negative relationship between the time necessary for germination and cyst age at activation is obvious for cysts 4 through 20 days old (Fig. 2a). Ultimate germination frequency was high for all four series, and was independent of cyst age on the time scale of this experiment. When the same germination data is plotted against an X-axis of cyst age (rather than incubation time), the cumulative percent germination is a clear function of cyst age alone (for cysts younger than 20 days old), and is independent of the age at which these cysts were activated (Fig. 2b).

In the same experiment, cysts stored at 3°C prior to activation showed much the same pattern of germination as those stored at 18°C. In fact, the median germination times (by definition, the time required for 50% germination) for cysts of the same age, stored at either temperature, were essentially identical (Fig. 3).

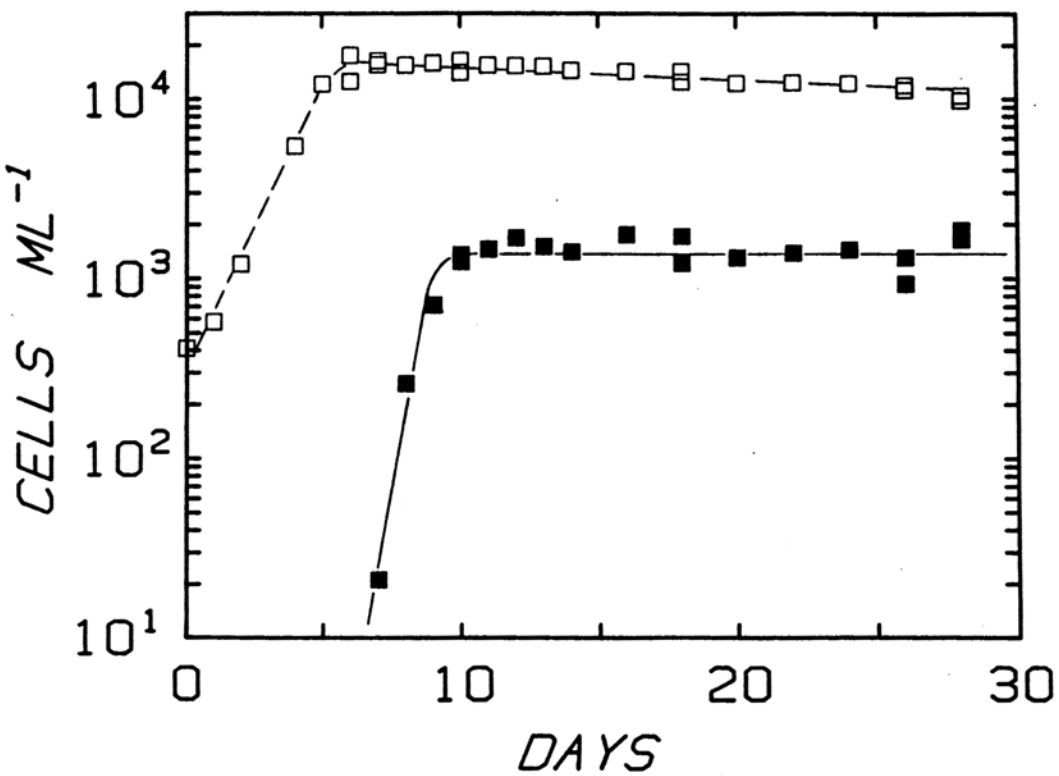


Fig. 1. Vegetative growth and cyst production by ScrpMxB in encystment-medium batch culture. Vegetative cells (□) and cysts (■) per ml; lines drawn by eye.

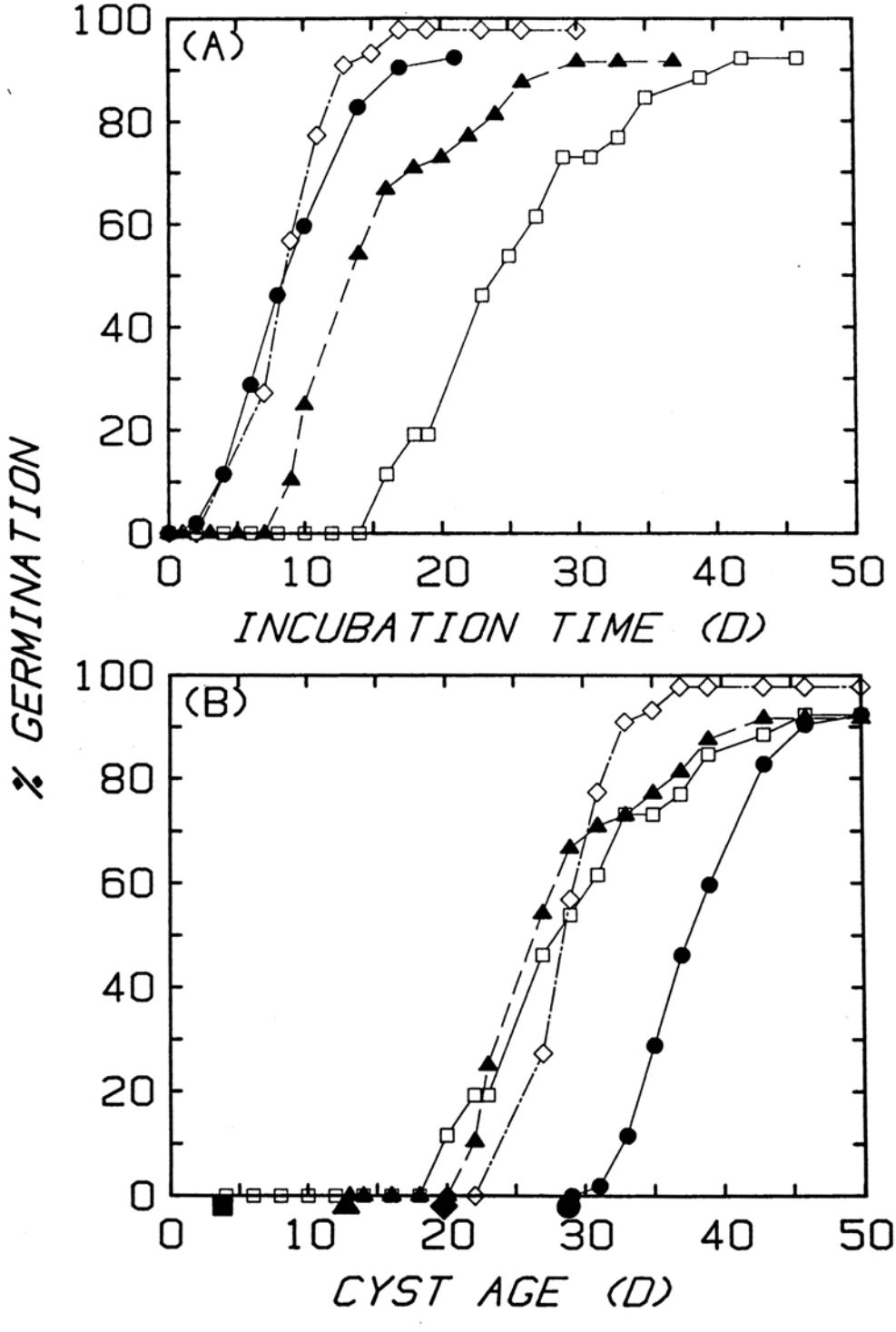


Fig. 2. Germination kinetics for cysts stored in light at 18°C for 4 (□), 13(▲), 20 (◇), and 29 (●) days prior to nutrient enrichment and shift to 15°C. (A): Data plotted against incubation time at 15°C. (B): Same data plotted against cyst age; enlarged closed symbols indicate the start of the 15°C incubation for each of the corresponding series.

This assessment of the effects of aging on germination can be extended by comparing both the median germination times and the ultimate germination frequencies observed among cysts of various ages, in all the sundry S. trochoidea germination experiments I have run to date (Figs. 3 & 4). Considered here are data from the treatments considered to be optimal for germination (i.e. nutrient replete, exposed to light, under permissive temperatures) within all the experiments from which the relevant parameters can be extracted.

Again, the negative relationship between germination time and activation age results in a minimum germination age of approximately 25 days (Fig. 3). For cysts older than 25 days, germination time appears to slowly decrease with increasing cyst age; a minimum germination time of 2 to 3 days is reached among cysts stored at 3°C for 75 days or longer (Fig. 3).

The ultimate germination frequency achieved in these experiments varied from 60 to 100%, with no clear pattern regarding cyst age or storage temperature (Fig. 4). The considerable variability in these data could obscure a subtle downward trend in germination frequency with increasing age; in any case, it is clear that a substantial proportion of cysts retain the ability to germinate after a year of storage at 3°C.

Temperature Effects. While the data presented above involves cysts stored at different temperatures, all were germinated between 15° and 18°C. To test the effects of temperature on germination, cysts

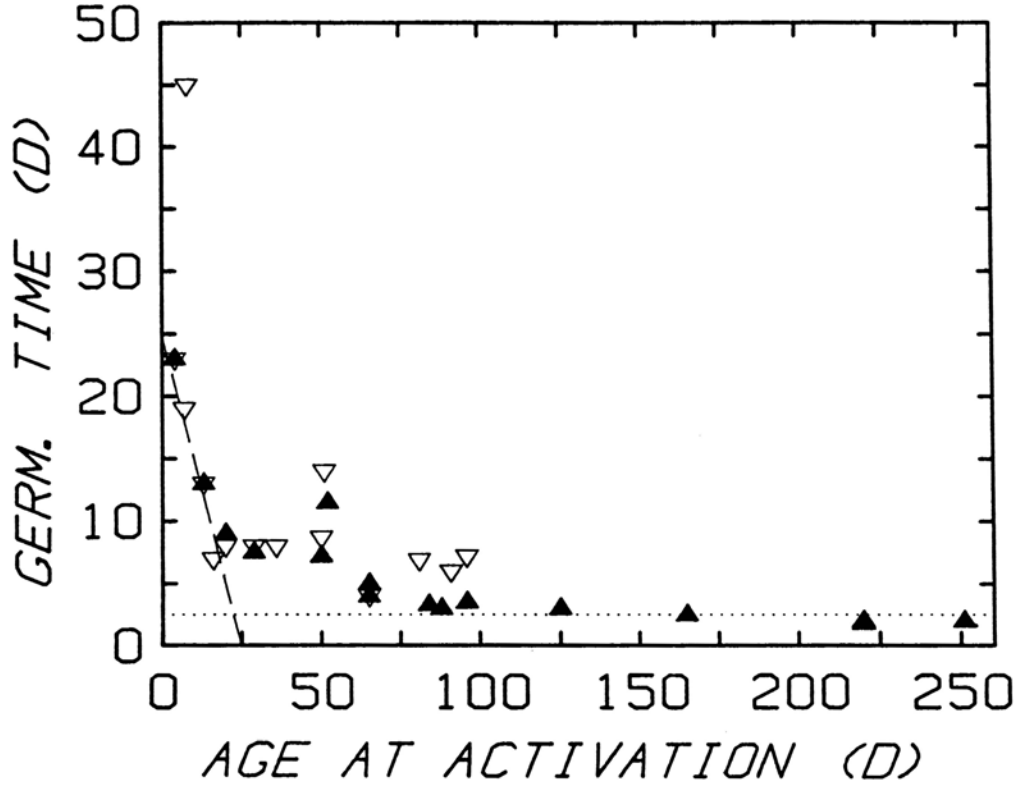


Fig. 3. Median germination time (after activation) versus cyst age. Data derived from all applicable S. trochoidea germination experiments run to date (see text). Closed symbols: cysts stored at 3°C prior to germination; Open symbols: cysts stored at 18°C. Broken line represents germination at a constant age of 25 days; dotted line represents a constant germination time of 2.5 days.

stored at 3°C in the dark were incubated at various temperatures in f/2 in the light (14:10 hr L:D), and scored for excystment over time (Fig. 5). For temperatures above approximately 14°C (up to at least 25°C) median germination time was 5 days or less, but it increased sharply at incubation temperatures below this range (Fig. 5a). Median germination time at 3°C was greater than 75 days (the final time point of this experiment); no germination had been detected in the 1°C treatment at that time. Except for these two lowest temperatures, ultimate germination success appeared independent of incubation temperature (Fig. 5a). Thus, while germination rate at 6°C was an order of magnitude slower than the optimum rate, the germination frequency finally achieved was not different from that at higher temperatures.

Cysts produced and stored at 18°C, and subsequently incubated in f/2 at the same temperature, germinated as well as those shifted down to 15°C (data not shown).

Vegetative growth rate was affected by temperature somewhat differently than germination (Fig. 5b). Maximum growth rate was achieved between 10°C and 20°C. Above this range growth rate fell off rapidly; no growth occurred above 23°C. In contrast, growth rate decreased gradually at temperatures below the optimum range, and was still 50% of the maximum at 2°C.

Medium Effects. Cysts stored undisturbed in their original cultures, under unchanged light and temperature conditions, do eventually germinate (Fig. 6). The final proportion of cysts germinating under these circumstances appears comparable to that achieved in parallel cultures spiked with nutrients, but the time

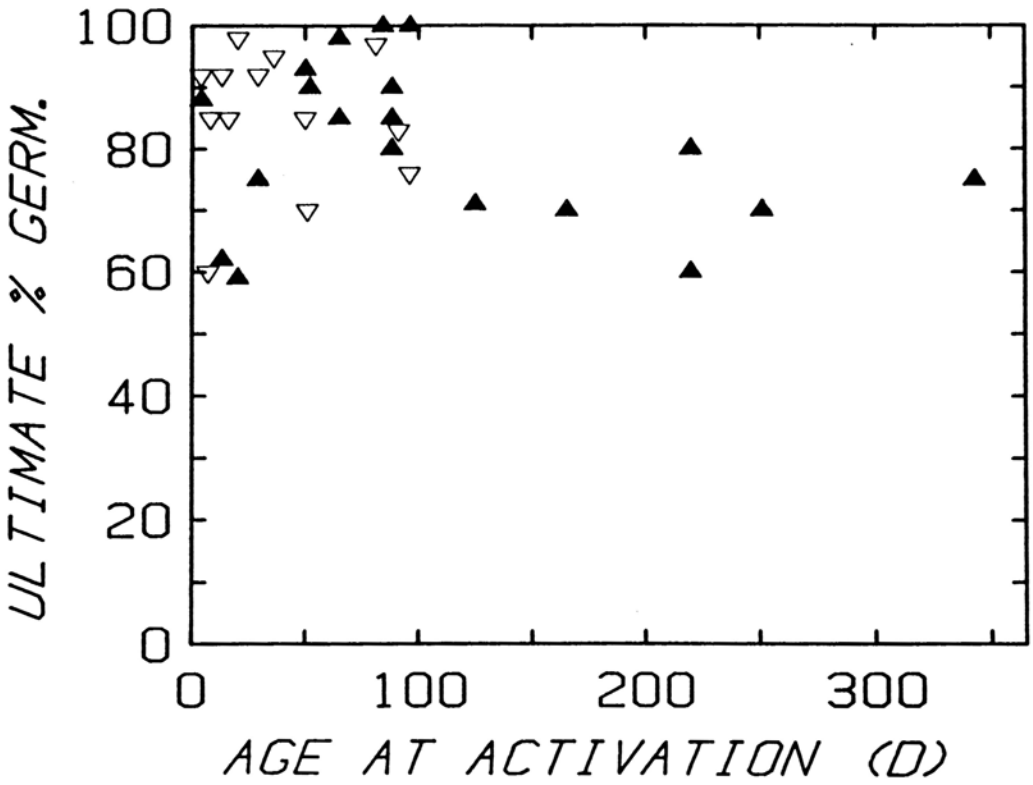


Fig. 4. Ultimate germination frequency versus cyst age. Data derived from all applicable S. trochoidea germination experiments run to date (see text). Closed symbols: cysts stored at 3°C prior to germination; Open symbols: cysts stored at 18°C.

required for germination is greatly increased. The pH of undisturbed cultures was not different from those enriched with nutrients.

Cysts harvested from culture and separated from vegetative cells prior to resuspension in unenriched Sargasso Seawater likewise showed a dramatic reduction in the rate of germination relative to f/2-enriched controls (Fig. 7). These cysts had just reached 50% germination at 65 days (the last time point of that series), while the median germination age was 22 days in the enriched controls. This relative reduction in germination rate in unenriched media was reflected in the dark treatments of this experiment as well (see below).

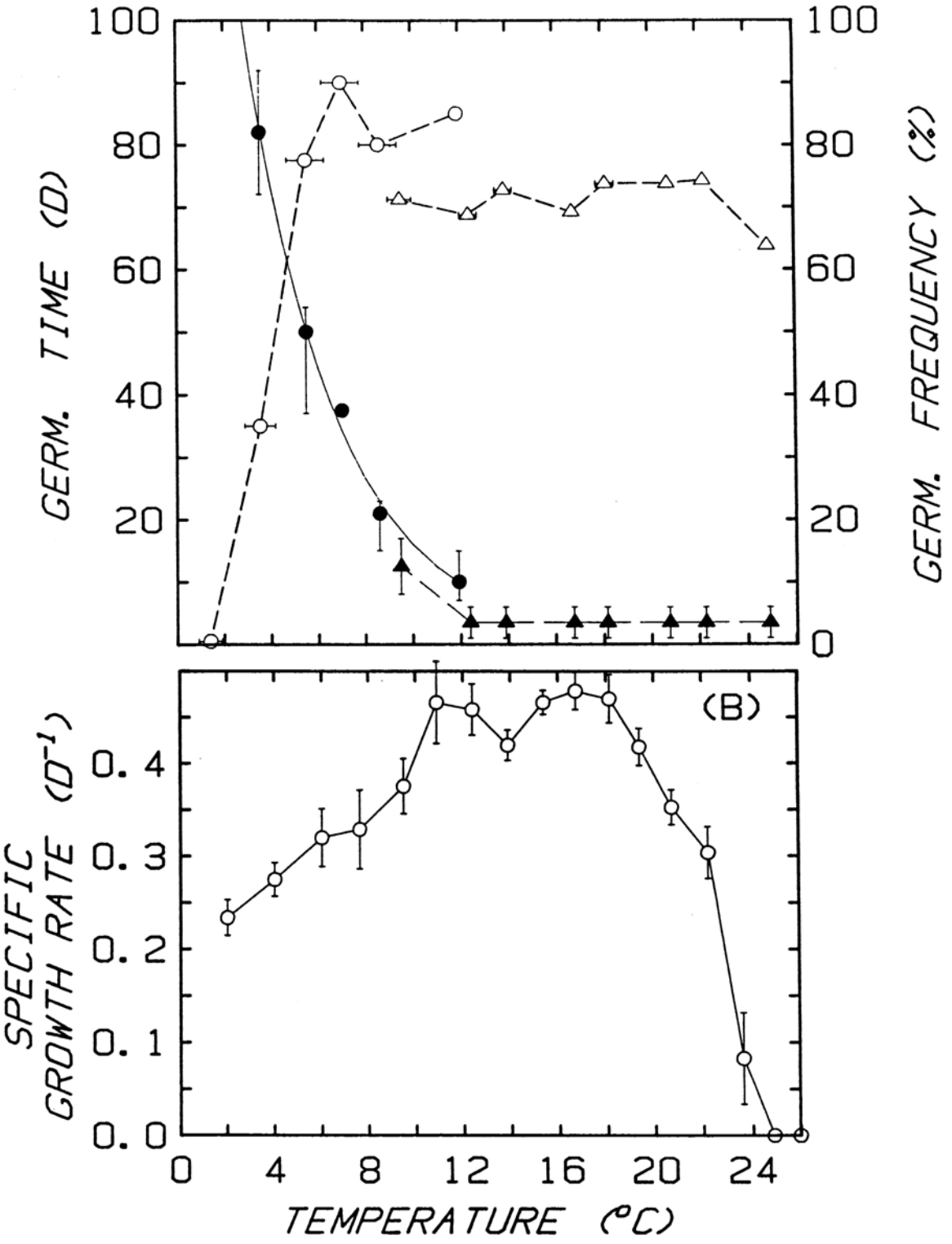
Low nutrient conditions do not always result in sub-optimal germination. Cysts stored in the dark at 3°C in their original cultures for 90 days, and then shifted to 18°C in the light with no added nutrients germinated comparably to identical cysts shifted to 18°C in the light with nutrient enrichment, although the final germination frequency in the latter case was slightly (though significantly) higher (Fig. 8).

Light Effects. The ability of cysts to germinate in the dark is problematic. Relative to germination under the standard 14:10 hr L:D regime, 90 day old cysts (previously stored in the dark at 3°C) which were incubated at 18°C in the dark (with f/2 nutrients) germinated poorly, achieving a final germination frequency of only 20% in 30 days (Fig. 8). However, in a subsequent experiment utilizing the same batch of cysts (now 250 days old), 60% germination was achieved in the dark after only 5 days (Table 1). Although this frequency of germination is significantly less than the mean 75% germination achieved in

light-exposed treatments ($P < 0.01$; ANOVA of arcsin-transformed data), it is still obviously much above the dark germination rate of 90 day old cysts. Among the light-exposed treatments in this experiment, germination frequency was independent of the length of the daily light period, within the range of 1 to 14 hrs per day ($P > 0.25$).

Relatively poor germination among young cysts in the dark is confirmed by the data in Fig. 7. In this experiment, cysts were stored at 18°C and germination in the absence of other changes (other than those incurred at the initiation of storage treatments) was monitored. In both the nutrient replete and unenriched dark treatments, a small proportion of cysts germinated immediately following the start of the experiment, after which no further germination occurred. Germination rate in the dark f/2 treatment was about 25%, while in the dark unenriched treatment it remained below 10%. Note that even after 395 days, no further germination was apparent among cysts stored under these conditions.

Fig. 5. Effect of temperature on S. trochoidea germination (A) and growth rate (B). (A): Median germination time (●,▲) and ultimate germination frequency (○,△) for cysts stored at 3°C (in the dark) prior to experiment. Two symbol shapes correspond to two separate expermiments. Median germination time estimated by linear interpolation of germination data at each temperature; vertical bars represent the resolution of these estimates (as determined by sampling schedual); horizontal bars show the the daily range in temperature. Solid line is the least squares fit of the experiment #2 germination times (●) to an exponential model ($Y= 205e^{-0.255(T)}$; $r^2= 0.994$). (B): Mean specific growth rate ± one SE, derived from 3- 10 independent estimates at each temperature.


100
80
60
40
20
0
GERM. TIME (D)
GERM. FREQUENCY (%)
(B)
SPECIFIC GROWTH RATE (D⁻¹)
0.4
0.3
0.2
0.1
0.0
0
4
8
12
16
20
24
TEMPERATURE (°C)

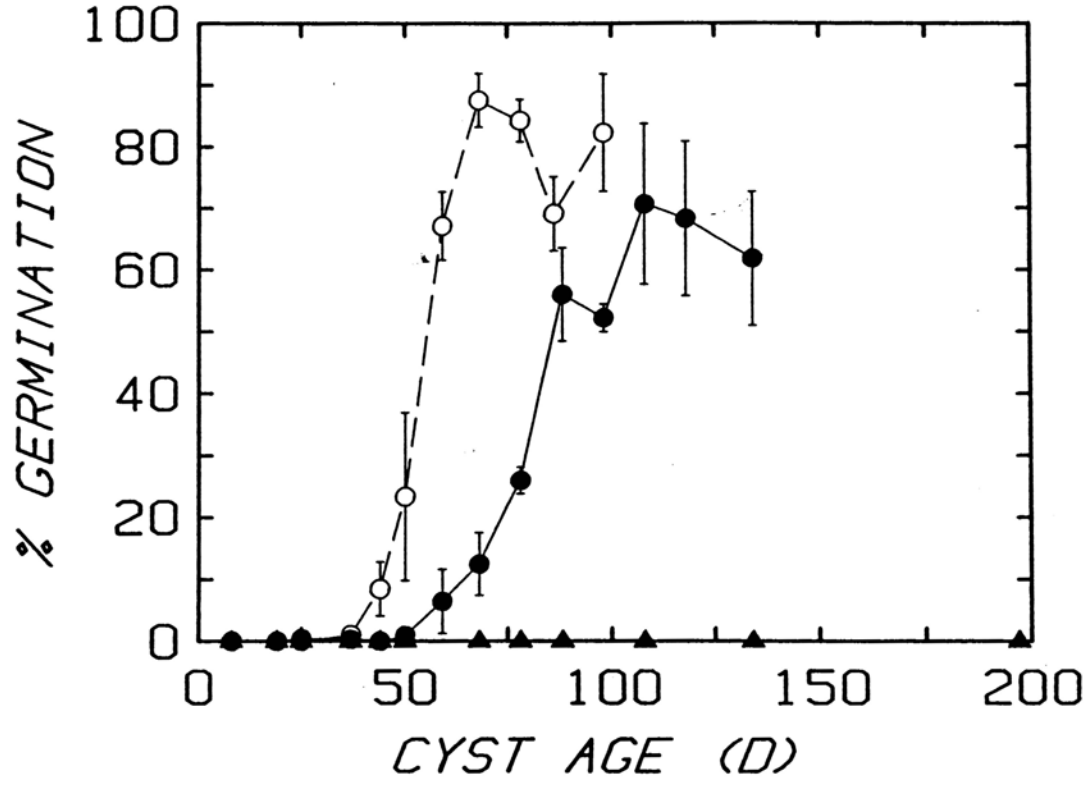


Fig. 6. S. trochoidea germination at 18°C, 14:10 hr L:D, in the absence of environmental changes (●), with nutrient enrichment (○); and at 3°C in the dark (▲). Means ± one SE (n=3).

DISCUSSION

Germination in Scrippsiella trochoidea cysts is regulated by a variety of environmental factors. The effects of these parameters can be manifested either as near-total suppression of germination, as was the case with light deprivation, or as changes in the kinetics of germination, as occurred with nutrient and temperature variations. This study is the first detailed investigation of the environmental regulation of germination in marine dinoflagellate resting cysts formed in culture and manipulated under controlled conditions. It is thus free from potential artifacts associated with sonication, isolation, and other techniques necessary for work with cysts recovered from natural sediments, and from complications arrising from the uncertain environmental histories of such cysts. The results provide valuable insights into the potential role of cysts in the ecology of this common marine dinoflagellate.

Encystment. Cyst formation in Scrippsiella trochoidea has been described by a number of authors (Braarud 1958, Wall et al. 1970, Watanabe et al. 1982). Watanabe et al. (1982) reported that these cysts are the product of sexual fusion, an observation I did not attempt to confirm. Both Baarud (1958) and Wall et al. (1970) noted that cysts appeared in batch cultures without specific manipulations or dramatic shifts in light or temperature. While my data confirm this observation, the fact that no cysts were observed in culture until after stationary phase had begun is inconsistent with the contention that cyst formation in S. trochoidea is favored by conditions optimal

for vegetative growth (Wall et al. 1970). Instead, it is likely that the onset of nutrient limitation is responsible for sexual induction and encystment in this species, as reported for a number of other dinoflagellates (Pfiester and Anderson 1986). My data do not address the effects of temperature and light on cyst production, but the published data for S. trochoidea, as well as other species, indicate that in the case of these environmental parameters, conditions condusive to encystment do roughly coincide with those conducive to growth (Watanabe et al. 1982, Anderson et al. 1984).

The number of cysts produced in my S. trochoidea cultures was approximately 10% of the maximum vegetative cell number, corresponding to a 20% rate of participation in sexual fusion (assuming that each cyst is a zygote, produced by the fusion of two gametic cells; Watanabe et al. 1982). This rate of gametic fusion is considerably less than the 60% reported by Watanabe et al. (1982) for the same species, but is similar to that observed in Gonyaulax tamarensis and Gyrodinium uncatenum cultures (Anderson et al. 1984, Anderson et al. 1985). The disparity between my results and those of Watanabe et al. (1982) is probably related to the different induction methods employed, although inter-clonal variability cannot be discounted (Pfiester 1975). The two clones are obviously different in other respects, particularly in their growth rate response to temperature (cf. Watanabe et al. 1982, my Fig. 5b).

As demonstrated by Anderson et al. (1985) for G. uncatenum, cyst production may not fully reflect the extent of gametic fusion in culture. In their cultures, a large proportion of the cells formed

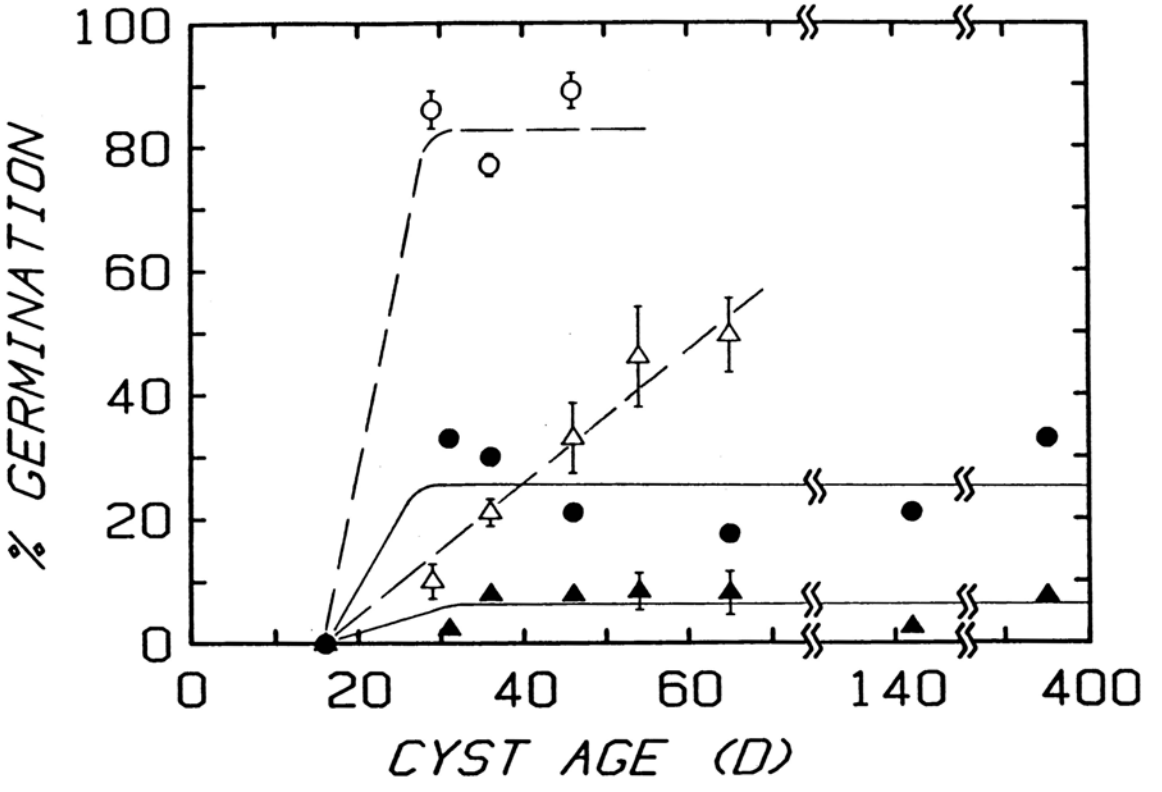


Fig. 7. Germination of S. trochoidea cysts separated from vegetative cells at age 17 d and incubated at 18°C in f/2, 14:10 hr L:D (○); in f/2, no light (●); in Sargasso seawater, 14:10 hr L:D (△); and in Sargasso seawater, no light (▲). Means ± one SE (n=3), except (●), which are unreplicated samples. Lines drawn by eye.

planozygotes but failed to encyst. Thus the relatively low final cyst yield in their study, and perhaps in mine, reflected the inadequacy of culture conditions for the transformation from planozygote to cyst, rather than for the transformation from vegetative to sexual reproduction.

Dormancy and Quiescence. Reference to dormancy is often made in discussions about dinoflagellate cysts, but the term is rarely defined, and the concepts associated with it remain vague. I will adopt the terminology used by Pfiester and Anderson (1986), wherein "dormancy" refers to a curtailment of germination as the result of an endogenous condition (i.e. as the result of some property of the cyst itself). The term "quiescence," on the other hand, will be applied when germination is inhibited by an exogenous, or environmental, factor. Thus dormant cysts cannot germinate, even under optimal environmental conditions, while quiescent cysts are competent to germinate but are inhibited from doing so by some environmental factor. For the purposes of this discussion, the terms "germination" and "excystment" are used synonymously.

Scrippsiella trochoidea cysts undergo a requisite period of dormancy lasting approximately 25 days (Fig. 3). During this interval, cysts placed under normally optimal germination conditions fail to germinate. Once the dormancy period is completed, cysts incubated under the same conditions germinate readily, while those placed under non-permissive conditions (see below) remain quiescent. Cysts have remained quiescent, but viable, for 340 days (at 3°C in the dark) as of this writing (Fig. 4), and they are fully expected to survive

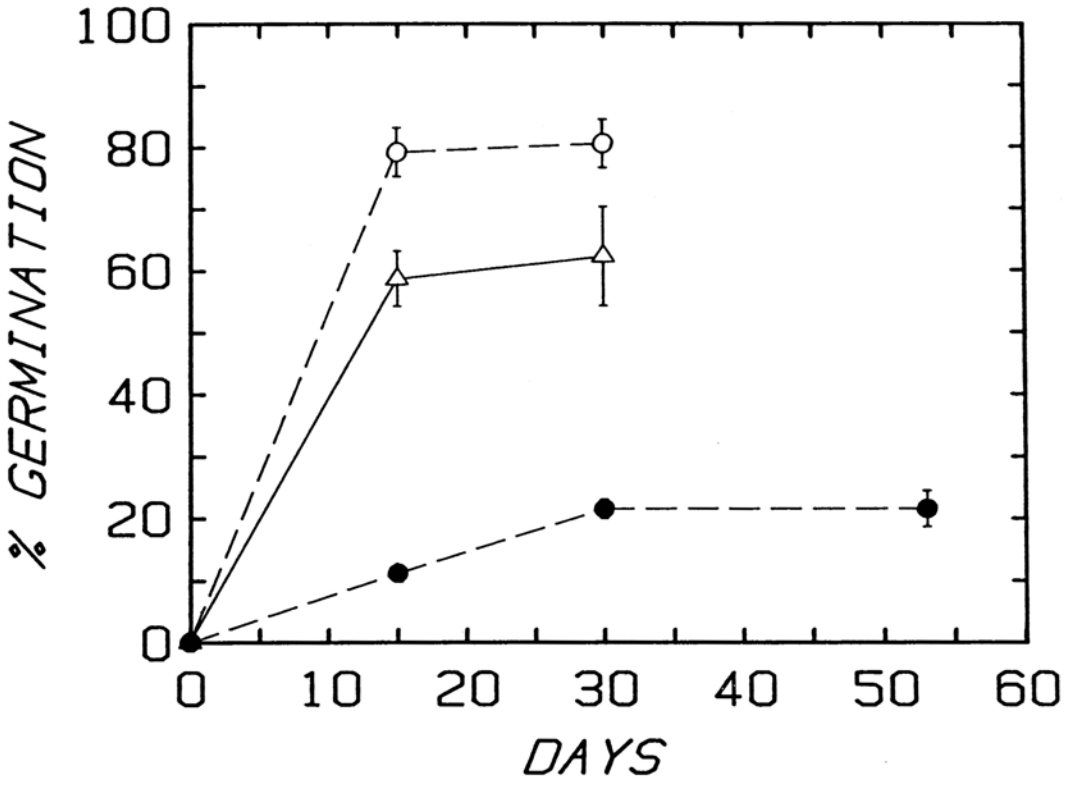


Fig. 8. Effect of darkness on S. trochoidea germination. Cysts stored at 3°C in the dark for 90 days prior to incubation at 18°C in f/2, 14:10 hr L:D (○); in exhausted culture medium, 14:10 hr L:D (△); and in f/2, no light (●). Means ± one SE (n=3).

considerably longer.

Analagous periods of dormancy have been observed in other dinoflagellate species (Dale 1983, Pfiester and Anderson 1986). Although the cysts of one species (Peridinium gatunense) can germinate within 12 hr of their formation (Pfiester 1977), most species undergo dormancy periods lasting from 7 to 20 weeks. Therefore the 3.5 week dormancy period observed for S. trochoidea is short relative to most dinoflagellates studied to date.

The duration of dormancy in a number species can be affected by the conditions under which cysts are stored. Particularly, a low temperatute treatment shortens the dormancy period in Peridinium cinctum (Dürr 1979) and results in more complete germination (once permissive conditions are restored) in Gymnodinium pseudopalustre and Woloszynskia apiculata (von Stosch 1973). On the other hand, low storage temperature lengthens the dormancy period in cysts of G. tamarensis and a Peridinium sp. (Anderson 1980, Endo and Nagata 1984). In contrast to these observations, temperature has no effect on the duration of dormancy in S. trochoidea. New cysts stored at 3°C or 18°C displayed the same 25 day delay in germination (Fig. 3).

The extent to which the dormancy period in S. trochoidea or other dinoflagellate species reflects a period of development (analogous to "morphological dormancy" in plant seeds; Nikolaeva 1977), or instead, an endogenous inhibition of an otherwise competent cell (analogous to "physiological dormancy"), is not known at present. A negative relationship between the length of the dormancy period and temperature, as reported for G. tamarensis, could be indicative of a

Table 1. The effect of photoperiod on germination frequency. Cysts stored previously at 3°C in the dark for 250 days. Germination scored after 5 day incubation at 18°C, in f/2, under the specified light regime. Differences tested by ANOVA of arcsine-transformed germination frequencies.

Photoperiod (hr dy^{-1})	Germination Frequency Mean (SE) [n=2]
14	79.0 (3.05)
8	73.3 (0.70)
1	72.4 (4.35)
0 (Dark)	58.8 (1.00)

(Differences among all groups):	$p < 0.05$
(among light treatments):	NS
(dark vs. light treatments):	$p < 0.01$

developmental requirement which takes longer to complete at lower temperatures (Anderson 1980, Dale 1983). By implication, those cases in which cold treatment improves germination should not involve such development, but rather some sort of endogenous inhibition, which is broken by cold storage. My observation that the duration of dormancy in S. trochoidea is independent of temperature obviously fits into neither of these catagories, and may well foreshadow the diversity of behaviors which might be observed as more dinoflagellate cysts are examined. Elucidation of the nature of dormancy in dinoflagellate cysts awaits detailed studies of the ultrastructure and physiology of these cells.

Excystment. Germination in S. trochoidea is influenced both by cyst age and environmental conditions. Excystment rate seems most sensitive to these factors, while with a few exceptions, the ultimate excystsment frequency is relatively stable. The effects of different environmental parameters are discussed below.

The relationship between median germination time and cyst age at activation is illustrated in Fig. 3. The data can be separated into three hypothetical developmental phases according to cyst age. For cysts within the first phase, from age 0 to approximately 25 days, the median germination time appears to be controlled strictly by cyst dormancy. The observed linear decrease in germination time within this interval is the mathematical consequence of a minimum germination age of approximately 25 days.

At the other extreme, cysts more than 75 days old appear to approach a minimum germination time of approximately 2.5 days. This

asymptotic value may be set by the absolute minimum time required to complete the physiological or developmental changes which underlie excystment.

The intermediate phase, between 25 to 75 days, appears to represent a transition from the dormancy period, when germination is impossible, to the final phase, when cysts are prepared to germinate optimally. Although variability in the data obscures the details of this phase, some process apparently continues in cysts that remain quiescent, resulting in increased germination competence in these cysts over time.

Spontaneous Excystsment. The requirement for a shift in environmental conditions to trigger germination has been mentioned in connection with a number of dinoflagellate species. However, the distinction between a requirement for an actual environmental change versus a requirement simply for a given set of permissive conditions has not yet been carefully examined (Dale 1983). Thus, while Anderson (1980) showed that an upshift in temperature from 4°C to 15°C or a downshift from 22°C to 15°C resulted in germination in G. tamarensis cysts, he pointed out that since no treatment involving both storage and germination at 15°C had been performed, he could not establish a need for a temperature shift, per se, as opposed to a simple need for incubation at 15°C. In fact, a number of dinoflagellates have been reported to germinate in the absence of obvious environmental changes, although quantitative data have not been presented (von Stosch 1973, Pfiester 1975, 1976, 1977, Pfiester and Skvarla 1979).

Scrippsiella trochoidea cysts do not require environmental shifts to initiate germination. Rather, my data are consistent with the concept of a permissive "window" of environmental conditions, within which non-dormant cysts will germinate, but outside of which such cysts remain quiescent (Dale 1983, Pfiester and Anderson 1986). Thus, cysts left undisturbed in their parent cultures, under unchanged light and temperature conditions (14:10 hr L:D, 18°C), did germinate (Fig. 6). Germination was significantly delayed relative to nutrient-enriched treatments, but the final proportion of cysts germinating in the two treatments was comparable. This experiment utilized separate culture tubes at each time point in order to avoid potential artifacts associated with repeated handling of the same tube over time. However, the possibility of other artifacts resulting from the presence of vegetative cells in the suspension (e.g. organic or inorganic nutrient release, oxygen concentration changes, etc.) cannot be discounted. These effects could be particularly pronounced within the pellets that accumulate at the bottom of culture tubes and which include all the cysts in addition to dead and scenescent vegetative cells. Therefore, while there is no significant doubt that temperature and light shifts are unnecessary as triggers of germination in S. trochoidea cysts, the conclusion that shifts in water chemistry are likewise not required must remain tentative.

Temperature Effects. Temperature is the environmental variable most often cited as controlling germination in dinoflagellate cysts (Dale 1983, Pfiester and Anderson 1986). Low temperature maintains quiescence in cysts of many species; a return to higher temperatures

appears sufficient to initiate germination. Few studies have addressed the effects of incubation temperature on germination in any detail. Huber and Nipkow (1923) reported that germination rate in Ceratium hirundinella varied with temperature in much the same way as it does in S. trochoidea (compare their Fig. 11 with my Fig. 5a). Within a certain temperature range, the time required for germination is at its minimum; beyond that range, germination time increases sharply. For C. hirundinella, this increase in germination time was observed above the optimum temperature range as well as below it; Scrippsiella trochoidea would probably show a similar increase above its optimal range, but no such increase was detected at the highest temperature tested (25°C).

It appears likely that the apparent decrease in "ultimate" germination frequency in S. trochoidea cysts at temperatures below 5 C is an artifact of the finite duration of the experimental incubations (see below). Decreases in germination frequency at non-optimal temperatures have been reported in Peridinium cunningtonii and a Peridinium sp. (Kadota et al. 1984, Endo and Nagata 1984). However, the lack of kinetic data in the first example, and the relatively short incubation time in the second, make it impossible to be sure that the observed relationship between germination frequency and temperature is not likewise a reflection of the effects of temperature on germination rate. In the present study, no significant germination was detected at 5-6°C until 25 days after the start of the experiment, yet germination at this temperature did finally reach a frequency of 75%.

The relationship between germination time and temperature in S. trochoidea and C. hirundinella suggests that for these species at

least, the permissive temperature "window" for germination does not have clearly defined boundaries outside of which germination is impossible. Instead, beyond the optimal temperature range, germination occurs at progressively slower rates. This reduction in the rate of germination, as reflected by an increase in median germination time, is well described in S. trochoidea by an exponential function, the slope of which corresponds to a Q_{10} of approximately 11 (Fig. 5a; r^2= 0.995, $p < 0.001$). Using this relationship, the extrapolated germination time for S. trochoidea cysts at 1-2°C is approximately 150 days, or twice as long as the duration of my temperature experiment. Thus, the possibility of germination at such low temperatures cannot be excluded. In this connection, note that in the present study the cysts reported to have remained quiescent for 340 days at 3°C were stored in the dark.

Although these data argue against a simple on/off response by cysts to temperature, the graded response observed is quite precipitous. Thus, although the germination rate at 3-4°C was estimated to be approximately 6% of the optimal rate, vegetative growth rate was reduced by only 50% at these temperatures (Fig. 5b). Furthermore, the Q_{10} values of 11 and 9 for germination rate in S. trochoidea and C. hirundinella, respectively, are far above the values reported for various metabolic processes in other algae (Soeder and Stengel 1974; Q_{10} for C. hirundinella germination calculated from data in Huber and Nipkow (1923) between 5°C and 20°C). Thus, while the response of cysts to temperature is not strictly an on/off one, neither does it appear to be a simple reflection of a general metabolic

slowdown with decreasing temperature. Rather, the steep slope of germination time versus temperature suggests the operation of a specialized control mechanism.

The optimal temperature range for germination in S. trochoidea does not coincide with the optimal range for growth (Fig. 5). Aside from the drastic reduction in germination rate at temperatures causing only moderate reduction in growth rate at the low end of the temperature range, large reductions in growth rate are apparent at the high end, above 23°C, while germination remains unaffected. Huber and Nipkow (1923) noted similarly that the optimal temperatures for excystment in C. hirundinella were higher than the optimal range for morphological development of the germling (21- 24°C vs. 15- 20°C, respectively).

Medium Effects. Nutrient concentrations and other water chemistry variables are not generally considered to exert significant influence on germination in dinoflagellate cysts. In fact, those few studies which have addressed the question have concluded that germination is insensitive to these variables, although subsequent survival and development of the germling may obviously be affected (Huber and Nipkow 1923, Anderson and Wall 1978, Anderson and Morel 1979). In contrast, the present study establishes that nutrient concentration can indeed influence germination in S. trochoidea cysts. In two experiments involving nutrient-enriched and unenriched media, germination was significantly retarded in the unenriched treatment. However, final germination frequencies appeared comparable, or only slightly reduced in the unenriched treatments (Figs. 6- 8).

Other factors can apparently modify these nutrient effects. The germination rate in cysts stored at 3°C in the dark for 3 months and then returned to 18°C (14:10 hr L:D) was comparable in enriched and unenriched treatments (Fig. 8). Furthermore, I consistently found that when cysts were individually isolated into tissue culture plates, germination in unenriched media was equivalent to that in enriched treatments (data not shown). Perhaps the environmental perturbations which are undoubtedly associated with such manipulations were responsible for stimulating germination in these treatments.

The inability of other studies to demonstrate nutrient effects may reflect real differences in germination behavior among dinoflagellate species. However, considering that the response reported here involved the kinetics of germination rather than ultimate germination frequency, and that this response could be overridden by other factors, the possibility of similar subtle effects of nutrient conditions on germination in the other dinoflagellate species examined cannot be excluded.

Light Effects. The finding that darkness may significantly reduce germination frequency in S. trochoidea is the first report of such a phenomenon in a dinoflagellate cyst. In most previous studies of other dinoflagellate species, comparable germination has been reported in the dark and in the light (Huber and Nipkow 1923, Anderson and Wall 1978, Krupa 1981, Hall 1982). An effect of darkness on cyst germination has been shown in two studies (Endo and Nagata 1984, Anderson in prep.), but in both cases germination rate was retarded, while ultimate germination frequency appeared not to be affected.

In contrast to these results, and to the temperature and nutrient responses discussed above, the response of S. trochoidea cysts to light conditions was manifested in the ultimate germination frequency rather than in germination rate. Thus, for cysts stored in the dark at 18°C, the maximum germination frequency of 25-30% was reached within 30 days, with no further increase apparent over the remaining 370 days of the experiment (Fig. 7). A 90 day cold dark pre-treatment, which had been sufficient to ameliorate the effects of nutrient deprivation on S. trochoidea cysts, was not sufficient to relieve the requirement for light (Fig. 8). However, 250 days of such pre-treatment was apparently effective: cysts thus treated germinated in the dark at 18°C only slightly less successfully than the light controls (Table 1).

Light could play two different roles in promoting excystment. First, it could provide energy, via photosynthesis, to drive the metabolic processes underlying excystment. Second, it might act as a trigger which initiates, but does not fuel, germination. Although these two hypotheses cannot be distinguished with the results in the present study, data published elsewhere (Chapt. 2) strongly favor the second, photomorphogenic, role of light in promoting germination in S. trochoidea.

The extremely low photon fluence threshold of this photomorphogenic response (Chapt. 2) indicates that while the brief, low intensity light exposures incurred at the initiation of my experiments are probably insufficient to provide cysts with any significant photosynthetic energy gain, they may be sufficient as

photomorphogenic triggers. The initial germination bursts observed in young cysts in "dark" treatments (Figs. 7 & 8) could be the result of such incidental exposures. Once the experiments were initiated, cysts would have experienced no further light exposure, hence those cysts not yet triggered would remain quiescent indefinitely (Fig. 7). The "dark" germination observed among 250 day old cysts (Table 1) could likewise be attributed to initial incidental exposure. A reduced light requirement (lower response threshold?) in older cysts could result in the higher germination frequency observed in this experiment. The absence of additional germination in Fig. 7 (subsequent to the initial burst observed) over 370 days of the dark incubation, argues against a complete disappearence of the light requiremnet in older cysts.

Confirmation of this hypothesis requires more experimental work, but if valid, it would help explain the disparity between my results and those published studies which report little influence of light on germination in dinoflagellate cysts. Again, the results could reflect real differences among different dinoflagellate species, but the possibility that low level light exposure may have stimulated germination in these studies, as I feel it did in the present case, cannot be ignored. Indeed, even if extreme precautions were taken to avoid accidental exposure during experimental manipulations (Chapt. 2), it would be hard to argue that any cysts recovered from the field had been exposed to <u>no</u> light prior to such manipulations. These problems notwithstanding, the data here, as well as those from previous studies, support the notion that <u>photosynthesis</u> is not a requirement for germination.

Ecological Implications. It is apparent that the germination behavior of S. trochoidea cysts is determined by no single environmental factor, but rather is the result of the interaction of several. In the present study, temperature, nutrient conditons, light regime, and cyst age all affected germination. The many unexpected interactions between these variables in terms of germination response argues for caution in using our laboratory results to predict cyst behavior in the natural environment where other variables may further complicate the picture. With this caveat in mind, some general implications of the present results to the ecology of Scrippsiella trochoidea can be discussed.

The relatively short dormancy period in S. trochoidea cysts, combined with the lack of a requirement for dramatic environmental shifts for germination, could facilitate a rapid cycling of the S. trochoidea population between its vegetative and dormant phases repeatedly during the year. Cysts of this species could thus be acting as mechanisms for survival of short term environmental adversity. Such a role in short term survival would be less appropriately applied to cysts of dinoflagellate species with longer dormancy periods. In this latter case, cysts might be more important in seeding one or two major blooms in a year (Anderson and Morel 1979).

Despite the ability of S. trochoidea cysts to support rapid population turnover, the quiescence of these cysts under cold, dark conditions is consistent with the longer term, over-wintering role often assumed for dinoflagellate cysts. Although these data suggest that germination might be possible even at very low temperatures, the

rate of such germination would be so low as to render it ecologically insignificant in many cases. For example, the extrapolated germination time of 5 months at 1-2°C is far longer than the 2 to 3 months during which such temperatures are actually experienced in local, well mixed waters. However, in deeper areas where low temperatures occur throughout the year, low temperature germination, no matter how slow, could be of great significance.

The significant retardation of germination in S. trochoidea cysts at lower temperatures (<10°C) which still support good vegetative growth could result in an apparently unnecessary delay (at least in terms of temperature tolerance) in seeding spring blooms of this species. Conversely, germination at high temperatures (>20°C) could release germlings into conditions which are unfavorable for growth. Thus, S. trochoidea cysts appear not to be finely tuned as "timing mechanisms" for bloom initiation.

The ecological consequences of the nutrient and light responses I observed remain poorly defined, as to some extent do the responses themselves. In general, benthic areas of cyst accumulation would be expected to be rich in nutrients, but until the response to nutrient conditions is more fully examined, no conclusions in this regard can be drawn. Likewise, a more complete description of light responses is necessary before their ecological implications can be drawn with any confidence. If the light requirement, whether photosynthetic or photomorphogenic in nature, is higher in younger cysts, the rapid population cycling suggested above could be greatly curtailed, particularly in deep areas and in cases of cyst burial. Furthermore,

the extent to which the hypothesized cold germination in S. trochoidea could occur at depth (where light is absent) depends upon the degree to which such a light requirement is relieved as cysts age.

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CHAPTER 2

Photomorphogenic Control of Germination in *Scrippsiella trochoidea* Cysts[†]

[†]The absence of "Introduction," "Methods," "Results," and "Discussion" sections in this chapter reflect the style of the journal to which the manuscript will be submitted. Methods are described to a large extent in the figure legends; further information can be found in Appendix II.

Although developmental responses to light are well known and ubiquitous among higher plants (Shropshire and Mohr 1983), examples of such responses among the algae are less common. Those responses which have been reported in this group are generally photoperiodic or require relatively long-term light exposures; most involve macrophytes (Dring and Luning 1983). This report documents a non-photosynthetic, low threshold, photomorphogenic[+] response in the marine dinoflagellate Scrippsiella trochoidea. In contrast to the reported behavior of other dinoflagellate species, I find that S. trochoidea resting cysts require light to germinate into motile, vegetative cells. This requirement is satisfied to a large extent by low photon fluences delivered in exposures as short as one second. Green light is most effective in eliciting the response. Given the primitive phylogenetic standing of dinoflagellates (Dodge 1983), and the relative rarity of green light mediated photomorphogenic responses in eukaryotes generally (Klein 1979, Tanada 1983), this phenomenon may hold considerable evolutionary and photophysiological interest, in addition to its more obvious ecological significance.

Temperature has been almost universally cited as exerting primary control over the germination of dinoflagellate resting cysts (Dale 1983, Pfiester and Anderson 1986). Most of the studies addressing the topic have found that cysts germinate comparably in the light or the dark (Huber and Nipkow 1923, Anderson and Wall 1978, Krupa 1981, Hall

[+]Throughout this thesis, I use the term "photomorphogenic response" to refer to a developmental response to light which is not mediated through photosynthesis.

1982). Two studies to date have demonstrated an effect of darkness on dinoflagellate cyst germination; both concluded that darkness delays, but does not prevent germination (Endo and Nagata 1984, Anderson in prep.).

I have confirmed that temperature can be important in controlling germination in cysts of S. trochoidea, but have also noted that germination in this species may be significantly reduced in the absence of light (Chapt. 1). The present study was undertaken in order to better define the influence of light on germination in this species.

Cysts for these experiments were produced under a 14:10 hr daily light-dark cycle at 18°C, in axenic clonal cultures (clone SA10, from Perch Pond, Falmouth, MA)(Chapt. 1). Within a week of the appearance of cysts, the cultures were enriched with inorganic nutrients at f/2 levels (Guillard and Ryther 1962) and placed in darkness at 3° or 18°C. Every precaution was taken to insure that no further light reached these cultures. Sampling and manipulations, when necessary, were carried out in total darkness.

Under these strictly dark conditions, cysts stored at 18°C failed to germinate over the 120 days of the experiment, although conditions should otherwise have been optimal for excystment (Fig. 1). Exposure of these cysts to the standard 14:10 hr L:D cycle (~650 µmol photons m^{-2} s^{-1}), at the same temperature, resulted in rapid germination.

The absence of germination among 3°C-stored cysts upon incubation at 18°C in the dark, as compared to the rapid germination among the same cysts illuminated daily for 14 hr, confirms the importance of

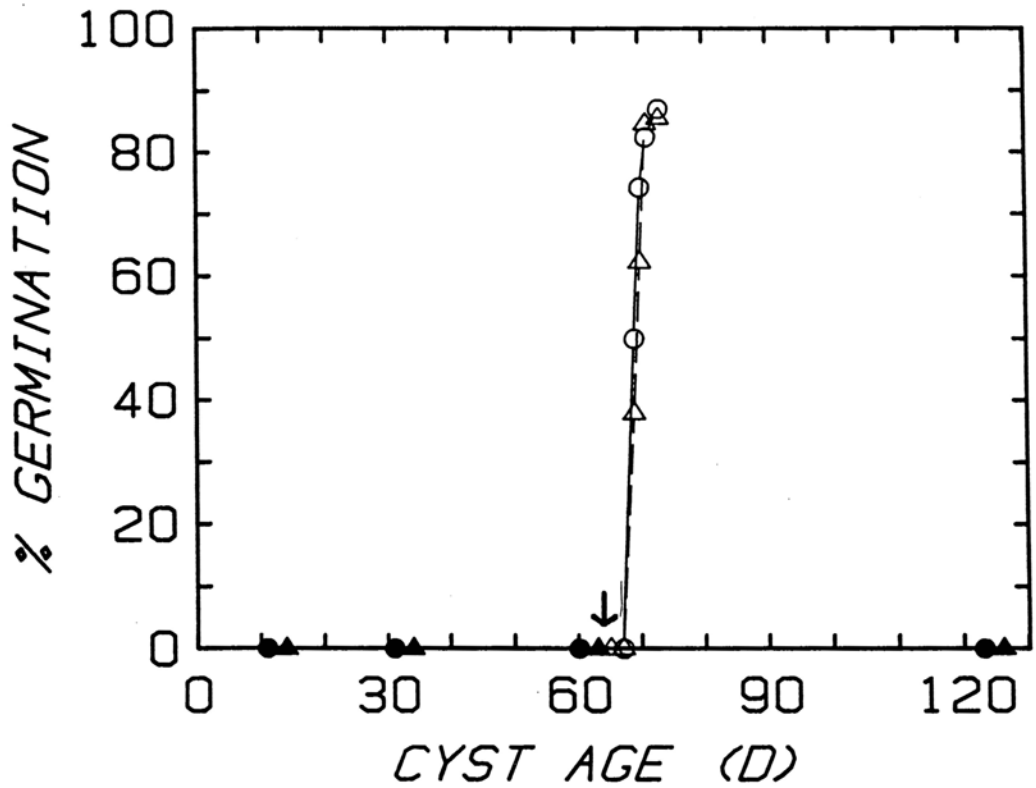

Fig. 1. Germination of S. trochoidea cysts placed in the dark at 3°C (●) or 18°C (▲) starting at age 7d. On day 65 (arrow), some cysts from both the 3°C and 18°C dark storage (○ & △, respectively) were placed under a continuing 14:10 hr L:D cycle at 18°C.

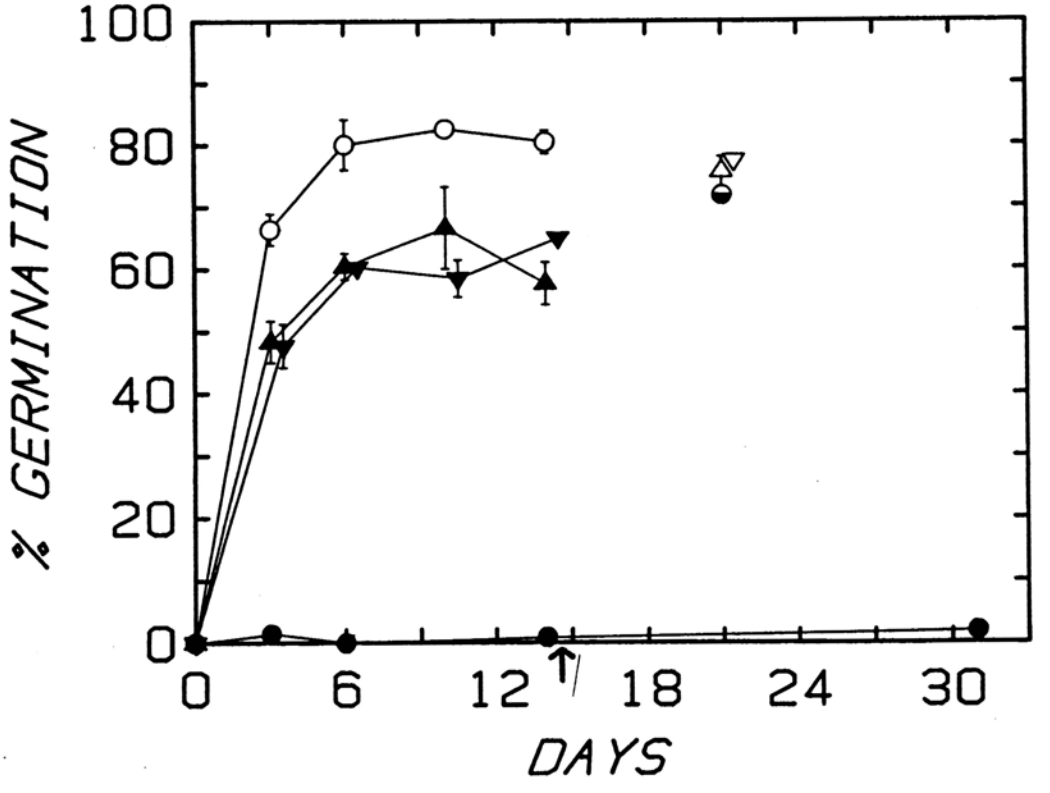

Fig. 2. Time course of germination by cysts (stored at 3°C in the dark) upon transfer to 18°C and exposure to a continuing 14:10 hr L:D cycle (○), to 60 minutes of light only (▼), to 2 minutes of light (▲), and to no light (●). On day 14 (arrow), cysts from the three latter treatments were exposed to the continuing 14:10 hr L:D cycle, and assessed for germination 6 days later (▽, △, & ◒, respectively). Means ± one SE (n=2).

light in controlling germination in this species (Fig. 2). Interestingly, cysts exposed to as little as 2 minutes of light germinated only slightly less successfully (though significantly so) than those exposed to the same level of illumination for 14 hr daily ($P < 0.001$). The response to 60 min of light was virtually identical to that in the 2 min treatment. In all cases, the ultimate frequency of germination was the responsive parameter; the rate at which this level of germination was reached appeared insensitive to light conditions.

These results clearly demonstrate that light is required for germination in S. trochoidea cysts. Furthermore, the fact that an exposure as short as 2 min is sufficient to stimulate germination raises the possibility that previous studies with dinoflagellate cysts (including my own), designed primarily with longer term light effects in mind, may have been incapable of distinguishing between such a low threshold response and true "dark" germination (Chapt. 1).

The relationship between total "white light" photon fluence and germination further underscores the sensitivity of S. trochoidea cysts to low levels of light (Fig. 3). A 50% response (based on a maximum achieved germination frequency of 60%) occurs in these cysts at approximately 0.2 μmol m^{-2} photon fluence. This photon fluence corresponds to an exposure time at standard culturing light intensities of far less than 1 second.

The observed germination response is dependent upon photon fluence (μmol m^{-2}), rather than fluence rate (μmol m^{-2} sec^{-1}) or exposure time, singly (Fig. 4). Note that equivalent germination is elicited by equal photon fluences, whether applied over 10 or 1000 s.

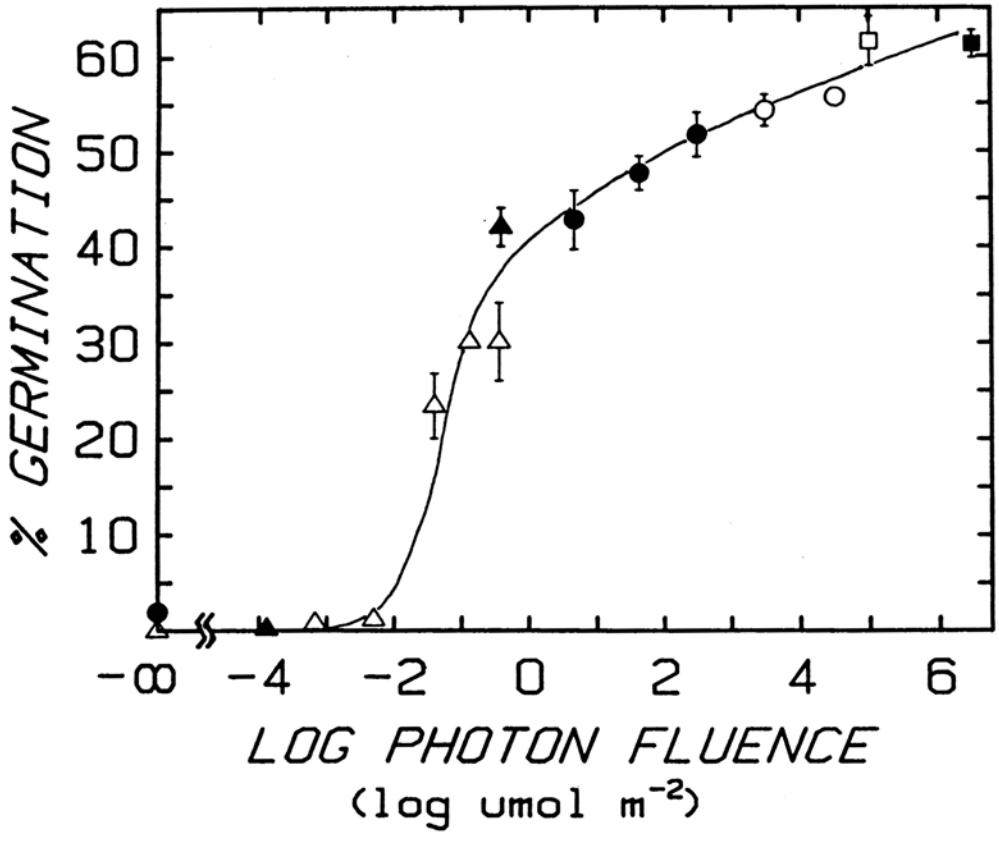

Fig. 3. Effect of "white light" photon fluence (μmol m^{-2}) on germination frequency. Incandescent source; except (□) and (■) (which are taken from Fig. 2), illuminated with a cool white fluorescent source. Photon fluence is the product of exposure time and fluence rate (measured for photosynthetically active wavelengths only [400nm< λ <700nm] with a scalar irradiance meter [Biospherical Instruments, Inc.]) and adjusted, as necessary, with neutral density filters. Exposure times are indicated by symbols: 1s (▲), 5s (△), 12s (●), 120s (□, ○), and 3600s (■). Means ± one SE (n=3, except n=6 for □ and ■). Line drawn by eye.

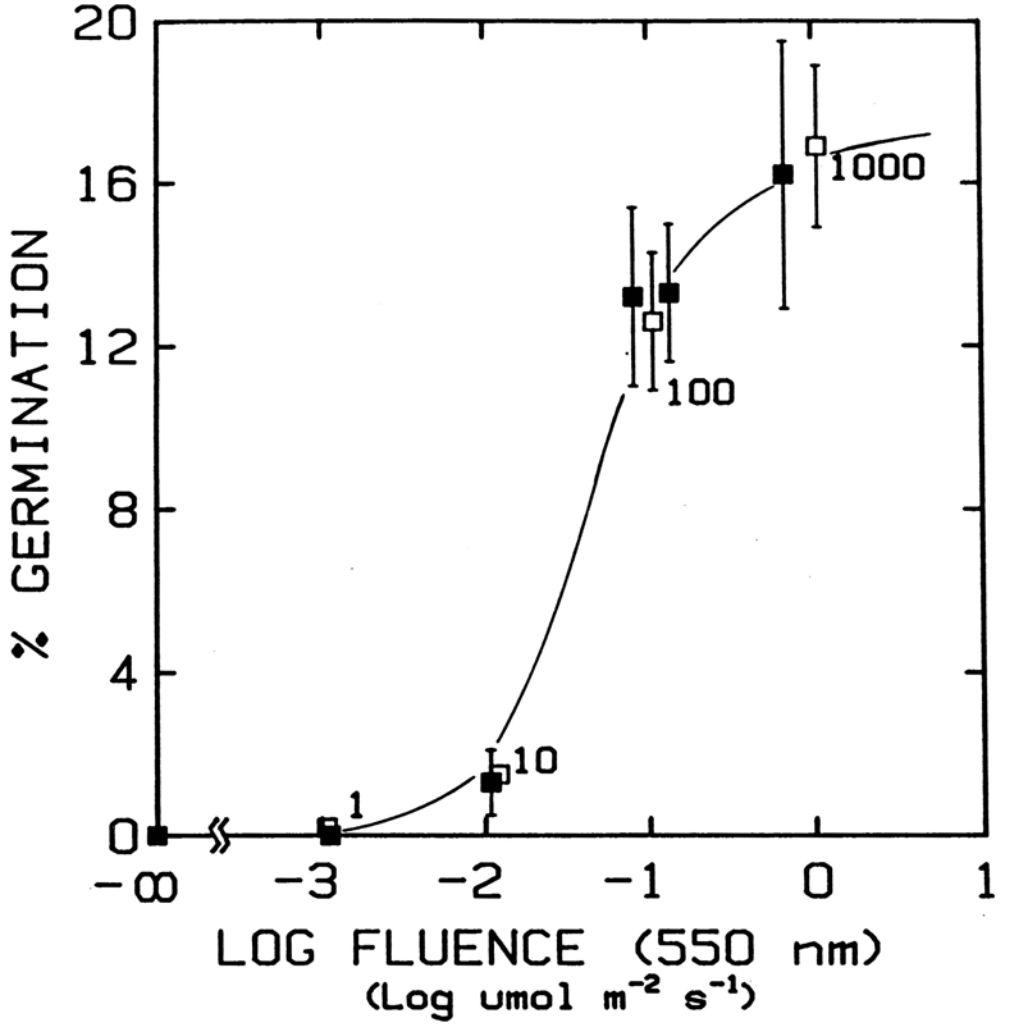

Fig. 4. The effect of photon fluence in the 550 ±20 nm wavelength band on germination frequency. Fluence was varied either by varying fluence rate (constant exposure time = 10 s) (■), or by varying exposure time (constant fluence rate = 1.06 x10^{-3} µmol m^{-2} s^{-1}) (□). Numbers indicate exposure times (s) employed in the latter group of treatments. Mean ± one SE; line drawn by eye.

The response by S. trochoidea cysts to the same photon fluence (0.12 μmol m^{-2}) at different wavelengths is shown in Fig. 5. Germination is maximal in the 550nm (yellow-green) band, with wavelengths above 620nm ineffective in stimulating a response at this fluence level. The response drops more slowly on the low side of 550nm, and is still significant in the blue (450nm) band. I found no evidence of significant modification of the 550nm band response by subsequent exposure to red or far-red light (650±20nm or 750±20nm) at equivalent fluence levels (data not shown). The relatively low germination frequency among all the wavelength-band treatments is consistent with the low response achieved in the white-light controls of this experiment (Fig. 5), and therefore cannot be taken to indicate a low response to monochromatic light generally. The cause of the reduced germination in this experiment is not known, but the pattern of response to different wavelength bands was confirmed by preliminary experiments which, however, involved less complete coverage of the spectrum.

Overall, the present data strongly suggest that the response to light in S. trochoidea cysts is not primarily photosynthetic. This conclusion is based on the low photon fluence requirement of the reponse, and its relative sensitivity to green light, as compared to blue or red. The conclusion is further supported by the inability of germinating S. trochoidea cysts to photosynthesize immediately following their activation (Chapt. 3). I cannot, however, exclude the possibility of photosynthetic involvement in the slight increase in germination frequency accompanying exposure to a continuous 14:10 hr

L:D cycle.

The only other published data concerning the effect of different wavelengths of light on dinoflagellate cyst germination is found in Huber and Nipkow (1923). As mentioned above, these authors reported that cysts of Ceratium hirundinella germinated equally well in diffuse daylight and in darkness. Also observed, however, was a retardation in germination among cysts exposed to red, yellow, green, or blue light. Interestingly, retardation was greatest in the blue and green treatments, the very bands most effective in initiating germination in S. trochoidea cysts. Unfortunately, lack of information regarding the actual spectral quality and photon fluence rates in their experiments makes it difficult to unequivically interpret Huber and Nipkow's work. In particular, the paradox that germination procedes optimally in white light or darkness, but is retarded by light of various colors (roughly covering the visible range), is hard to resolve.

Other non-photosynthetic responses to light among the dinoflagellates include phototaxis (Halldal 1958, Hand et al. 1976, Forward 1970) and growth inhibition by far-red exposures (Lipps 1973). With one exception, the active wavelength bands for these phenomena are blue, red, and far-red; the photoreceptor systems responsible are therefore almost certainly different than the one involved in the light-triggered germination of S. trochoidea cysts. In contrast, Halldal (1958) reported a phototactic response in Prorocentrum micans whose maximum sensitivity at 570 nm is suggestively close to the 550 nm maximum observed here. However, in the same study S. trochoidea phototaxis was found to be maximally responsive around 475 nm, with no

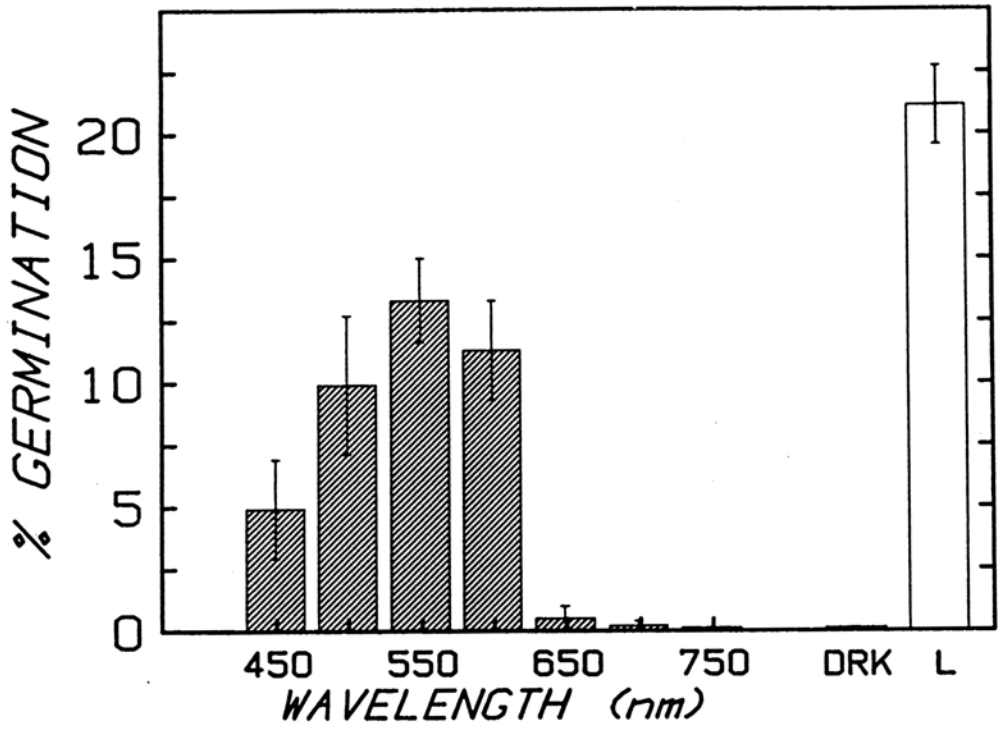

Fig. 5. Germination response of S. trochoidea cysts to wavelength. Hatched bars show the frequency of germination achieved in cysts exposed to equal photon fluences (~0.12 μmol m^{-2}) in 7 different wavelength bands. Incandescent source, in combination with wide band interference filters (40 nm half power band width, blocking outside of band better than 0.1% between low U.V. and 1000nm; Ditric Optics Inc.). Exposure time was 10 s in all cases; fluence rate was adjusted with neutral density filters and by varying source voltage. Means ± one SE (n=3); bar width represents half power band width, on wavelength scale shown. Open bar is the "white" light control: unfiltered incandescent source, 4.2 μmol m^{-2} (corresponding to approximately 0.5 μmol m^{-2} in the 550±20nm band). "DRK" bar is the dark contrl.

response occurring above 520 nm. This latter response to wavelength represents the more common situation for algal phototaxis.

Among the algae generally, germination of cyanobacterial akinetes and diatom resting spores has been widely found to be light-dependent (Yamamoto 1976, Braune 1979, Hollibaugh et al. 1981, Chauvat et al. 1982, Hargraves and French 1983). In contrast to the present case however, the response in akinetes is maximal in the red wavelengths and generally requires extended exposures (hours to days) and high photon fluences (Braune 1979, Reddy and Talpasayi 1981). Likewise, diatom resting spores are responsive only to relatively high fluence rates (Hollibaugh et al. 1981). In view of these differences it is unlikely that the requirement for light in akinetes or diatom spores is evolutionarily homologous to that in S. trochoidea cysts. Many of the physiological and ecological consequences of these phenomena, however, could well be analogous.

To what extent could a light requirement with the general properties described here control the germination of S. trochoidea cysts in nature? The answer will of course depend upon a number of factors, including the optical properties of overlying waters, the extent to which reciprocity in the response holds (i.e. the maximum time over which "photon counting" can occur), and the influence of cyst age and environmental parameters on the response itself. Despite these complications, we can gain some insight into the possible consequences of the phenomenon by calculating theoretical "critical" depths at which certain response levels would be expected (Table 1).

The fluence rates required for 50% and 10% germination were

Table 1. Theoretical critical depths for 50% and 10% germination among S. trochoidea cysts in different coastal water types. Based on transmission within the 550±20nm band; assumed surface irradiance in that band = 1.15×10^2 µmol m^{-2} s^{-1}. See text for details.

Jerlov Water Type	Transmittance (550nm)	Critical Depth 50% Germination	Critical Depth 10% Germination
1	86.5 %/m	59 m	116 m
3	81.0	41	80
5	72.0	26	51
7	63.0	19	37
9	53.0	14	27

calculated using the data in Fig. 3 and assuming 1) that 30% of the visible quanta from the white light source were active (this is a liberal estimate); and 2) that cysts are capable of integrating photon flux over 1000 s (this is a minimum estimate derived from the data in Fig. 4, and is probably very conservative; e.g. see Luning and Neushall 1978). To calculate critical depths, 1) a 40 nm wavelength band centered at 550 nm was assumed to contain all the active quanta (again, a conservative assumption - see Fig. 5); 2) the downward irradiance within this band was taken to be 1.15×10^2 µmol m^{-2} s^{-1} at the sea surface (from Jerlov 1968, for the Baltic Sea at high solar elevations); and 3) extinction coefficients were assumed constant over depth, and assigned the values given in Jerlov (1968) for different water types.

The results of these calculations indicate that in the most turbid coastal water (type 9), there is sufficient light at 14m to result in 50% germination among S. trochoidea cysts (Table 1). In clear coastal waters, the same response could occur at 40 to 60 m, while the maximum depth for 10% germination in such waters is greater than 100 m.

It therefore appears that in deep coastal and shelf waters, cysts could be deposited at depths great enough to prevent germination through light limitation. In such cases, resuspenion from the sediment surface and advection to shallower depths would presumably be required before germination could occur. On the other hand, in shallower habitats cyst germination is less likely to limited by light. Thus, the rapid cycling of S. trochoidea populations between vegetative and dormant stages, as hypothesized in Chapt. 1, remains plausable in these

habitats. Note, however, that burial of cysts in the sediment could undoubtedly result in light limiting conditions regardless of the depth of the overlying water column. In that case, again, resuspension from the sediments would necessarily precede germination.

Although the ecological consequences of light-triggered germination in S. trochoidea cannot be fully appraised without further information regarding the physiology of the response, it is clear that light can no longer be ignored in discussions concerning the behavior of dinoflagellate cysts in the natural system.

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Chapter 3

Biochemical Composition and Metabolic Activity of *Scrippsiella trochoidea* Cysts

INTRODUCTION

Many dinoflagellates are known to produce cysts during their life history (Dale 1983, Pfiester and Anderson 1986). These cysts are commonly assumed to represent "resting" stages which serve as mechanisms for survival during periods of environmental adversity. Dramatic morphological differences between the encysted and vegetative stages within a single dinoflagellate species, and the extended longevity of cysts under environmental conditions unsuitable for vegetative growth, argue strongly that significant physiological differences between the two cell types should exist. This hypothesis has never been directly tested, however. In this study, the gross biochemical composition and metabolic activity of cysts of the marine dinoflagellate Scrippsiella trochoidea are examined and compared with those of vegetative cells.

Our present knowledge regarding the composition and metabolic activity of dinoflagellate cysts comes from microscopic and ultrastructural studies of these cells. Bibby and Dodge (1972) reported that significant ultrastructural changes accompanied encystment in Woloszynskia tylota. Cysts of this species contained large vacuoles and a pigmented accumulation body (of unknown composition), and many small lipid bodies. Lipid globules were also apparent within cyst chloroplasts and the thylakoids of these chloroplasts appeared abnormally aggregated. Furthermore, the cytoplasm lacked golgi bodies and other membranous components and took on a granular appearance. These observations suggested that lipids

accumulated within cysts of this species as storage compounds, and that photosynthesis and general metabolic activity in these cells were reduced.

Similar observations of lipid and starch accumulation, granular cytoplasm, reduced pigment content, and chloroplast reorganization have been made in cysts of a number of other dinoflagellate species (Wall and Dale 1969, von Stosch 1973, Anderson 1980, Chapman et al. 1982).

No quantitative data regarding the composition or metabolic activity of dinoflagellate cysts are currently available. I have previously described the environmental and biological control of dormancy and germination in Scrippsiella trochoidea cysts (Chapt. 1 and 2); I now present data concerning some of the gross biochemical and metabolic changes which underly these processes.

METHODS

Cyst production, storage, and germination. An axenic clone of Scrippsiella trochoidea, designated SA10, was used throughout this study. Isolation of this clone and maintainance of cultures was as described previously (Chapt. 1).

Cysts were produced in 1/10 h/2 batch cultures (Guillard 1975) of SA10, at 18°C under a 14:10 hr daily light-dark cycle (cool white fluorescent lamps, approximately 450 $\mu E\ m^{-2}\ s^{-1}$). For the compostion time course, 140 25-ml cultures were innoculated from the same cell suspension at the same time and incubated as above. Culture growth and encystment was monitored on a daily basis in a number of those tubes. Within 12 days of the first appearance of cysts, the cultures were enriched to 1/10 h/2 nutrient levels and placed in darkness at either 3°C or 18°C. No germination occurs under these storage conditions (Chapt. 2). Germination was induced by incubation of the cultures at 18°C under the standard 14:10 hr L:D cycle.

Sampling. At each sampling time point, three culture tubes from each treatment were harvested; these represented the three replicates for that treatment in all subsequent analyses. Three aliquots of GF/F-filtered Vineyard Sound seawater ("VSSW") were carried through the entire harvesting procedure on each sampling day, and later served as blanks in all analyses.

After resuspending any accumulation of cells at the bottom of the cultures with a glass rod, tubes were vortexed thoroughly and the contents of each transferred to two 15-ml conical-bottomed glass

centrifuge tubes. These suspensions were underlaid with 0.5 ml Percoll-sorbitol solution (Price et al. 1978; Chapter 1) and centrifuged at 600 xg for 20 min in a horizontal rotor. Vegetative S. trochoidea cells are retained at the Percoll-seawater density discontinuity, while cysts are pelleted to the bottom of the tube. The supernatant plus density interface (including the vegetative cells) was transferred into another centrifuge tube, and the remaining pellet (containing cysts) was resuspended in 5 ml VSSW. The two supernatants and pellets thus derived from each original culture tube were combined to form the "vegetative" and "cyst" fractions for that culture. Cyst samples were re-purified in another Percoll gradient, as above, and the pellets resuspended in 5 ml VSSW. Finally, all tubes were centrifuged as above, aspirated to approximately 0.1 ml, and resuspended in 2.0 ml VSSW.

At this point, 100 µl was removed from each tube for cell sizing (see below), and two 50 µl aliquots were withdrawn for cell counts. These 50µl samples were added to 450 µl VSSW and preserved with 10 µl 1/4 strength Utermöhls solution (Utermöhl 1958). Cell counts were made on 100 µl aliquots from each of these suspensions in a Palmer-Maloney slide at 125x magnification.

The remaining sample was filtered at low vacuum (<2.5 cm Hg) onto 13mm ashed, CM-rinsed GF/C glass fiber filters. After the sample tube was rinsed twice with VSSW, the filter was carefully removed from its holder and stored in 2:1 (v/v) chloroform/methanol ("CM") at -20°C in a teflon-capped borosilicate vial, the headspace of which was flushed with N_2 prior to sealing.

For cell volume estimates, the unpreserved 100 µl sample was briefly spun on a bench-top centrifuge, and the pellet resuspended in 50 µl VSSW. A 25 µl aliquot was loaded under a coverslip (supported with bits of clay to avoid cell distortion) and cells were photographed at 200x magnification. Measurements of cell length and width (excluding the thecae) were subsequently made on these photomicrographs. Volumes were calculated for at least 40 cells from each treatment assuming an ellipsoidal cell shape.

Biochemical Extraction. Samples were analyzed for chlorophyll-a ("chl-a"), lipid, carbohydrate, and protein following a sequential extraction scheme adapted from Li et al. (1980). The reader is referred to Roberts et al. (1955), Sutherland and Wilkinson (1971), and Hitchcock (1983) for extended discussion of the rational and relative merits of such schemes (see also Appendix II).

Sample filters were carefully transferred to 12 ml graduated teflon-capped centrifuge tube tissue grinders (Bellco Glass, Inc., Vineland, NJ), and ground with glass pestles by hand for 2 minutes. Microscopic examination revealed that this treatment disrupted 98% of the cysts originally present. The pestles were rinsed clean into the same tube with CM, after which the solvent from the storage vial was added. Samples were then centrifuged at 2000 xg for 20 min at room temperature in a horizontal rotor. The supernatent was saved in another graduated centrifuge tube, while the pellet was resuspended in a few ml of CM rinsed twice from the original sample vial, and centrifuged again, as above. The supernatent from this step was combined with the first, and represents the "CM fraction." This

fraction includes lipids, chl-a, and low molecular weight compounds.

The CM-extracted pellets were resuspended in about 1.5 ml 5% (w/v) trichloroacetic acid (TCA), incubated at 90°C for 20 min, and centrifuged as above. The supernatant was set aside as the "TCA fraction," containing carbohydrates. In contrast to the results of Hitchcock (1983), who reported that only a small proportion of the total carbohydrate in Gonyaulax tamarensis was solublized in hot TCA, I found that the recovery of particulate carbohydrate in this fraction was approximately 78% for vegetative cells and 90% for cysts of S. trochoidea (Appendix II). Nevertheless the carbohydrate values reported in this study are the sum of the TCA and NaOH fractions.

Finally, the TCA-extracted pellets were resuspended in 2 ml 0.1 N NaOH and digested for 20 min at 90°C. Neither increased NaOH concentration nor increased digestion time resulted in increased protein liberation (Appendix II). Samples were centrifuged, and 1.5 ml of supernatent was removed and saved as the "NaOH fraction," containing protein and some residual carbohydrate. This fraction was neutralized with 150 µl 1N HCl prior to analysis.

Analyses. Chlorophyll-a and pheophytin were measured in the CM fraction fluorometrically (Strickland and Parsons 1972) using a Turner Designs model 10 fluorometer (excitation filter #10-250, emission filter #10-051; Turner Designs, Mountain View, CA). The assay was calibrated with extracts from exponentially growing S. trochoidea cells, the chl-a content of which was measured spectrophotometrically using the equations given by Parsons and Strickland (1963). The recovery of chl-a in CM was not significantly different than that in

90% acetone (Appendix II). Prior to determining that acidification had no detectable effect on the subsequent recovery of lipid from the CM fraction (Appendix II), I did not measure pheophytin in addition to chl-a (this is true for all vegetative cell samples, and cyst sample days 21-78). Therefore, for Fig. 1 no correction for pheophytin was employed. Note that because exponentially growing cells have negligable pheophytin (data not shown), the differences between exponential-phase cells and stationary-phase cells or cysts shown in Fig. 1 represent conservative estimates. For comparisons among cysts, a constant pheophytin/chl-a ratio was assumed for days 21- 78 (0.43 and 0.24 for cysts stored at 18°C and at 3°C, respectively), based on the measured ratios on days 101 and 141 of the time course, and on the initial days of the cyst germination experiment. The alternate assumption of equal pheophytin/chl-a ratios at both temperatures does not qualitatively change the relationships shown in Fig. 2 and Table 1.

Lipids were assayed gravimetrically after two Bligh and Dyer (1959) extractions of the CM fraction as follows. To the CM fraction was added 0.25 volumes of 0.9 g l^{-1} $MgCl_2 \cdot 6H_2O$ solution; the samples were mixed well, capped tightly, and kept below 5°C for at least 60 min. They were then centrifuged at 5°C for 20 min. (2000 xg) to insure complete phase separation, and the upper phase plus the interface aspirated and discarded. The samples were evaporated to dryness under N_2 at approximately 40°C, redissolved in 1.6 ml CM, and extracted again as outlined above. The lower (chloroform) phase from this second extraction was transferred to a vial, from which 100 µl

aliquots were repeatedly loaded into a pre-weighed aluminum weigh-boat and evaporated on a warm hot-plate. Generally, a total of 500 µl was used for each sample. Finally, the evaporated samples were weighed to the nearest 0.1 µg on a micro-gram balance (Perkin Elmer AD-2 Autobalance). VSSW blanks, carried through the entire sampling and extraction procedure, were subtracted from the sample values.

Protein was measured in the NaOH fraction with an adaptation of Bradford's (1976) dye binding assay (Spector 1978). One ml of the coomassie brilliant blue solution (Biorad Laboratories, Richmond, CA) was added to duplicate 200 µl aliquots of the neutralized NaOH fraction, vortexed, and the absorbance at 595 nm read within 15 minutes. For each analytical run, 25 µl of a protein standard in distilled water was added to 800 µl of VSSW blank and analyzed. Bovine serum albumin was employed as the standard, but the protein values reported are normalized to their bovine gamma globulin equivalents, as the response to this protein by the assay is considered more generally representative.

Carbohydrate was measured both in the TCA and NaOH fractions using the phenol-H_2SO_4 assay of Dubois et al. (1956). Duplicate or triplicate 400 µl aliquots were combined with 400 µl of phenol solution (5%) in 13x100 mm disposable borosilicate tubes. Two ml of concentrated H_2SO_4 was then added, and the tubes vortexed. Absorbance at 485nm was measured at least 1 hr later, in the reaction tubes. Glucose standards, in the appropriate VSSW solutions, were used to calibrate the assay.

Metabolic Rates. Photosynthesis and respiration were measured as

oxygen production or consumption in a water jacketed Clark-electrode incubation chamber (Rank Bros., Cambridge, England) similar to the one described by Delieu and Walker (1972). Illumination was provided by a 150 watt incandescent flood lamp; light intensity was controlled with layers of black nylon screening, and by varying the lamp voltage. Light intensity was measured with a scalar irradiance meter (Biospherical Instruments, Inc., San Diego, CA), emersed in seawater in a beaker whose outside diameter was equivalent to the outside diameter of the incubation chamber. The electrode was calibrated using N_2-flushed and air-saturated seawater as end points. Oxygen production or consumption rates were derived graphically from the chart recorder output, and were corrected for oxygen consumption by the electrode (determined on sterile solutions before and after each run). Preliminary experiments with vegetative S. trochoidea cells showed that O_2 consumption or production remained linear for at least 2 hr under the conditions of my experiments.

For the germination experiment, cysts (which had been stored for over 3 months at 3°C in the dark) from 20 culture tubes were aseptically harvested, combined in a single 2.5 ml suspension in f/2 medium (Guillard and Ryther 1962), and incubated at 18°C under a 14:10 hr L:D cycle. Photosynthesis and respiration in this suspension were measured at the same time (about the middle of the light period) on each day. Immediately prior to these measurements, the suspension was freed of any vegetative cells with a sterile Percoll step-gradient (Chapt. 1) and resuspended in fresh f/2 medium. Thus the measured rates reflect the metabolic activity of encysted cells only. After

this separation, the cysts were loaded into the electrode chamber and incubated at 18°C for 30 min in darkness, followed by sequential 30 min periods of light (260 μE m^{-2} s^{-1}), dark, and light. Oxygen consumption or production became linear within the first 5 min of each incubation period, and remained constant for the remainder of that period. Mean respiration and photosynthetic rates were calculated using the two measurements made during the dark and light incubations on each day, respectively. Incubation of cysts at higher light intensities did not result in an increase in oxygen production, indicating that the measured photosynthetic rates were light-saturated. Following these measurements, the cyst suspension was recovered from the electrode, and its incubation under the standard L:D cycle continued. Examination of the cyst suspension at the close of the experiment using epi-fluorescent microsopy (DAPI-stained samples; Porter and Feig 1980) failed to reveal significant numbers of bacteria relative to sterile controls.

Statistical Analysis. Two-way ANOVA was employed to analyze the long term trends in cyst composition, taking time and temperature as the two treatment variables. As this approach necessitates a balanced design, the initial point (day 21) is excluded from this analysis. The F-max and Kolmogorov-Smirnov tests confirmed that sample variances were homogeneous and that sample values were distributed normally (Sokal and Rohlf 1981). Rates of carbohydrate loss were estimated with least squares linear regression, and the significance of the slope tested within the regression ANOVA.

Among the measured rates of respiration and photosynthesis,

significance was tested within the appropriate ANOVA, employing a-priori pair-wise daily comparisons of oxygen consumption in the dark vs. the electrode blank, and of net oxygen consumption in the dark vs. net oxygen consumption in the light, respectively. Standard errors for gross photosynthesis and P/R values were derived from error propagation calculations (Peters et al. 1974), for which the error-MS term from the ANOVA was taken as the within-sample variance, and its degrees of freedom (10 in this case) taken as the degrees of freedom associated with the SE estimate.

For calculating respiration rates from carbohydrate and lipid data, complete oxidation of substrate via the standard respiratory pathways was assumed (Lehninger 1975). Thus, the ratio of µg substrate consumed to µmol O_2 produced was taken as 30 and 11 for carbohydrate and lipid, respectively.

RESULTS

Cysts and vegetative cells of S. trochoidea differ significantly in their biochemical composition (Fig. 1). In particular, late exponential-phase cells contain 3 times more protein per cell, and 4 times more chl-a, than do newly formed cysts. On the other hand, the carbohydrate content of these cells is an order of magnitude less than that of cysts. Lipid concentrations in both stages are about equal.

Stationary-phase cells of S. trochoidea are considerably smaller than exponential cells (cell volumes = 2300 and 5900 μm^3, respectively); however the per-cell composition differences between these two cell types is not solely the result of cell volume differences. The composition of stationary-phase cells, when normalized to cell volume, appears to be intermediate between exponential-phase cells and cysts with regard to protein, carbohydrate, and chl-a (Fig. 1b). The lipid content of all three cell types was comparable on a per volume basis. For comparative purposes, twice the (per cell) stationary-phase composition is indicated in Fig. 1a, since each cyst is assumed to be formed by the fusion of two gametic cells.

Changes in biochemical composition were monitored over time for cysts stored in the dark at 3°C and at 18°C (Fig. 2). Cysts stored at 18°C contained significantly less lipid and chl-a, and significantly more carbohydrate over the course of the experiment than those stored at 3°C (Table 1). Protein content was not affected by storage temperature. Note that all the cysts for this experiment were produced in the same cultures at the same time, and therefore must have

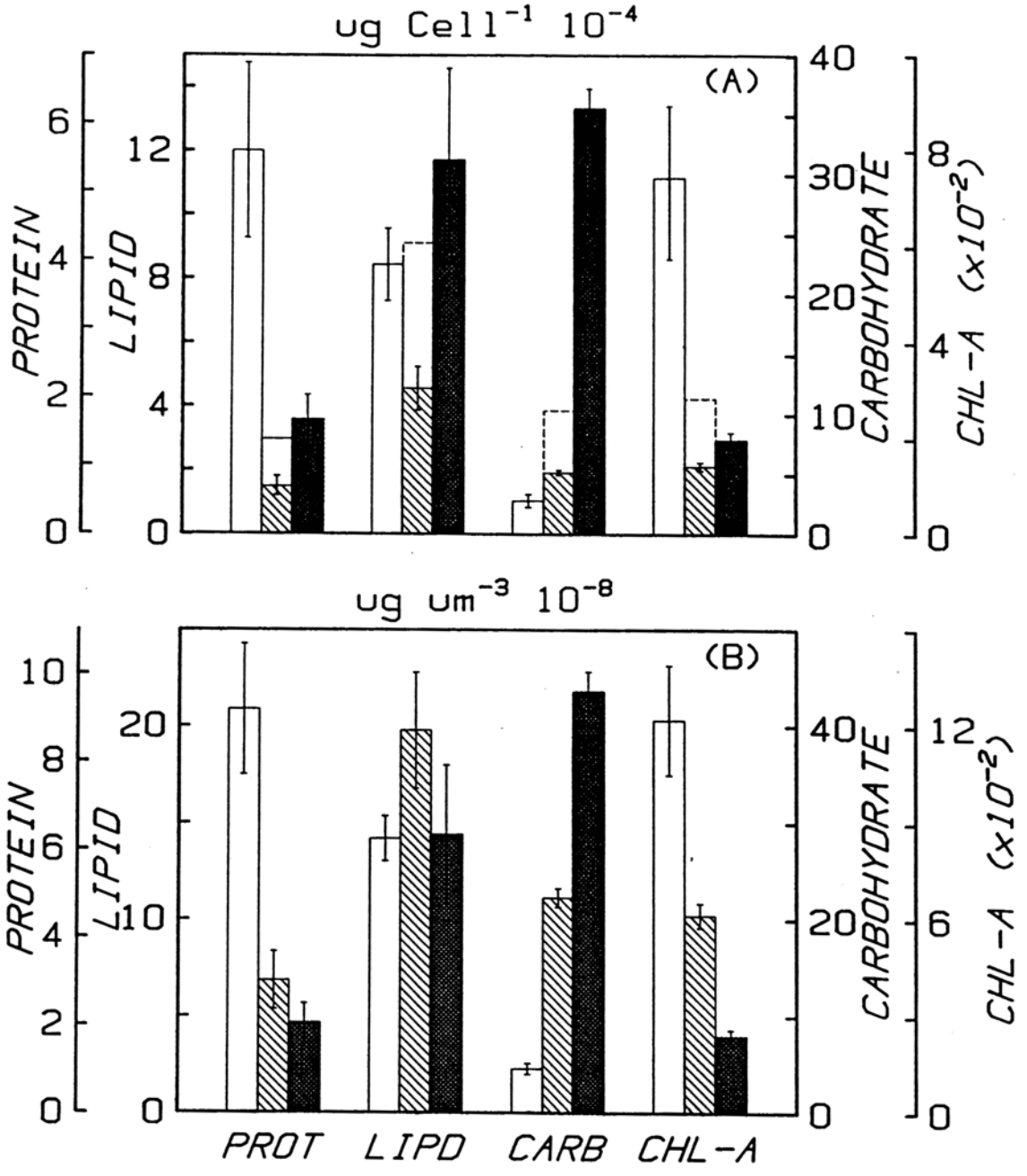


Fig. 1. Biochemical composition of late exponential-phase cells (open bars), stationary-phase cells (hatched bars), and newly formed cysts (dark bars). A) Composition on per cell basis; broken-line bars represent twice the stationary-phase cell composition (see text). B) Composition normalized to cell volume. Mean ±SE (n=3).

Table 1. Statistical analysis of changes in biochemical composition of quiescent cysts over time. Cysts stored at 3°C or 18°C in the dark. Two-way ANOVA of the data from Fig. 2, days 49-141 (see text).

	Factor		
Constituent	Time	Temperature	Interaction
Protein	*	NS	NS
Lipid	*	***	NS
Carbohydrate	***	***	*
Chl-a	***	***	NS

NS = P> 0.05
* = P< 0.05
*** = P< 0.001

initially had identical biochemical composition.

Storage time exerted a statistically significant effect on all four parameters (Table 1). Since the initial composition sample (day 21, Fig. 2) is not included in this analysis (see Methods), this time effect is independent of those changes which occurred withing the first 28 days of storage. The significance of the effect of storage time on protein content is clearly the result of the single low protein values from day 141 (Fig. 2B), and must therefore remain suspect. However for lipid and chl-a, the time effects are reflected as monotonic upward or downward trends, respectively. Only in the case of carbohydrate is the effect of storage time different at different temperatures (i.e. the interaction term for this parameter is significant [Table 1]). Thus, from day 49 through 141, cabohydrate in 3°C-stored cysts decreased steadily, while that in 18°C-stored cysts dropped only very slowly, if at all (Fig. 2E).

Germination was initiated by incubating cysts (stored at 3°C in the dark) at 18°C under a 14:10 hr L:D cycle (Fig. 3). Upon activation, the rate of carbohydrate loss increased by greater than 10-fold relative to that in unactivated cysts. This change in rate occurred within 12 hr of activation; the rate of carbohydrate loss remained high thereafter, through at least 48 hr. Within the first 24 hr, cyst protein began to increase; it more than doubled by 72 hr. After a lag of 24 to 36 hr, chl-a content increased dramatically, reaching a maximum at 72 hr of five times its initial level, and then declining to near initial levels by 120 hours. Lipid content did not change significantly over the course of the experiment. Note that by 120 hr,

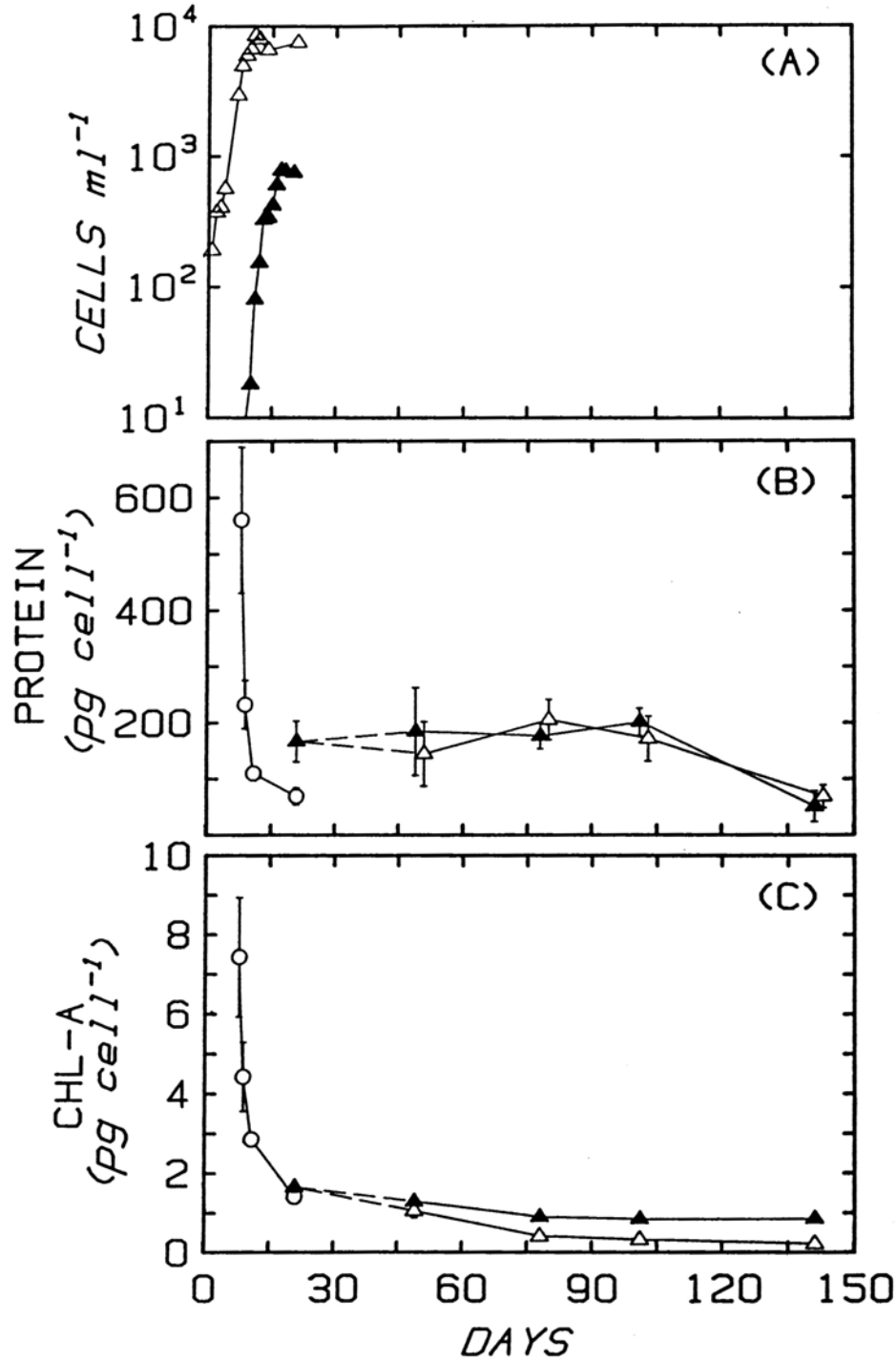


Fig. 2. Changes in biochemical composition in cysts and vegetative cells over time. A) Culture growth and cyst formation; vegetative cells (△) and cysts (▲). B) through E) composition over time; vegetative cells (○), cysts stored at 18°C (△), cysts stored at 3°C (▲). Cyst age = 0 on day 15; storage treatments initiated on day 21. Mean ±SE (n=3).

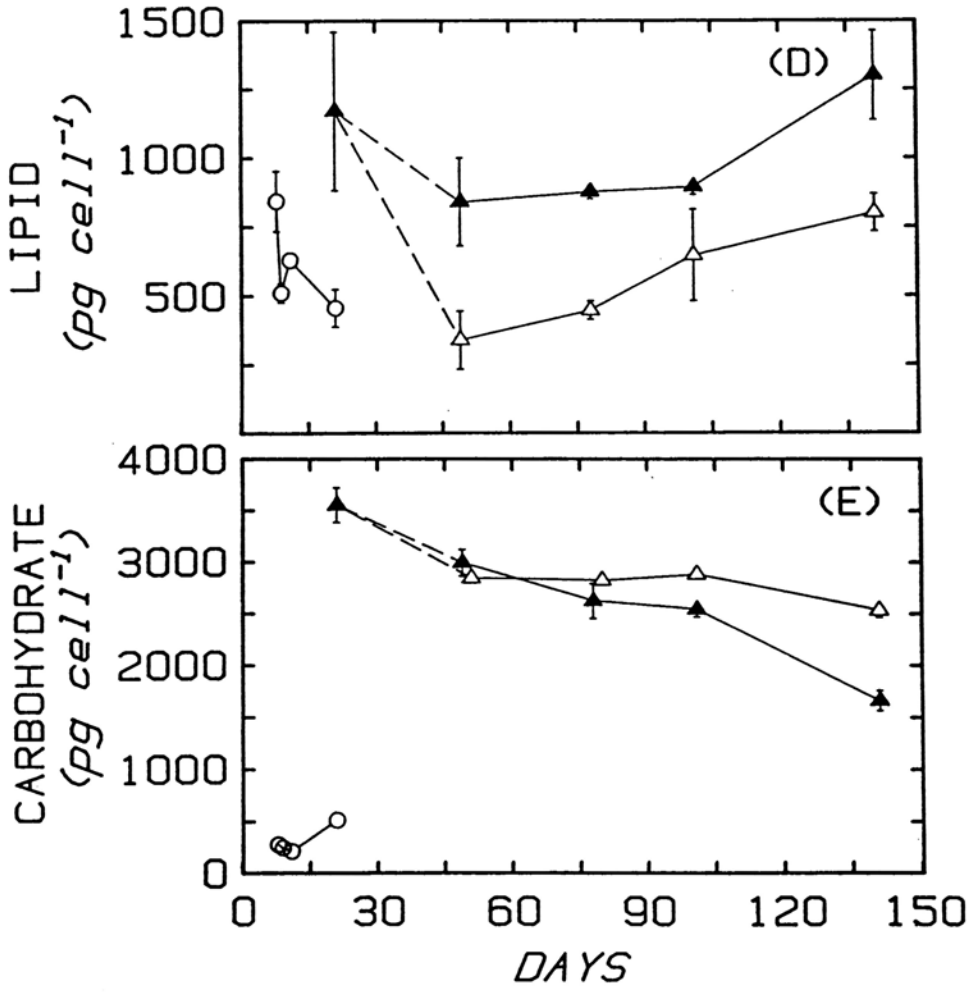
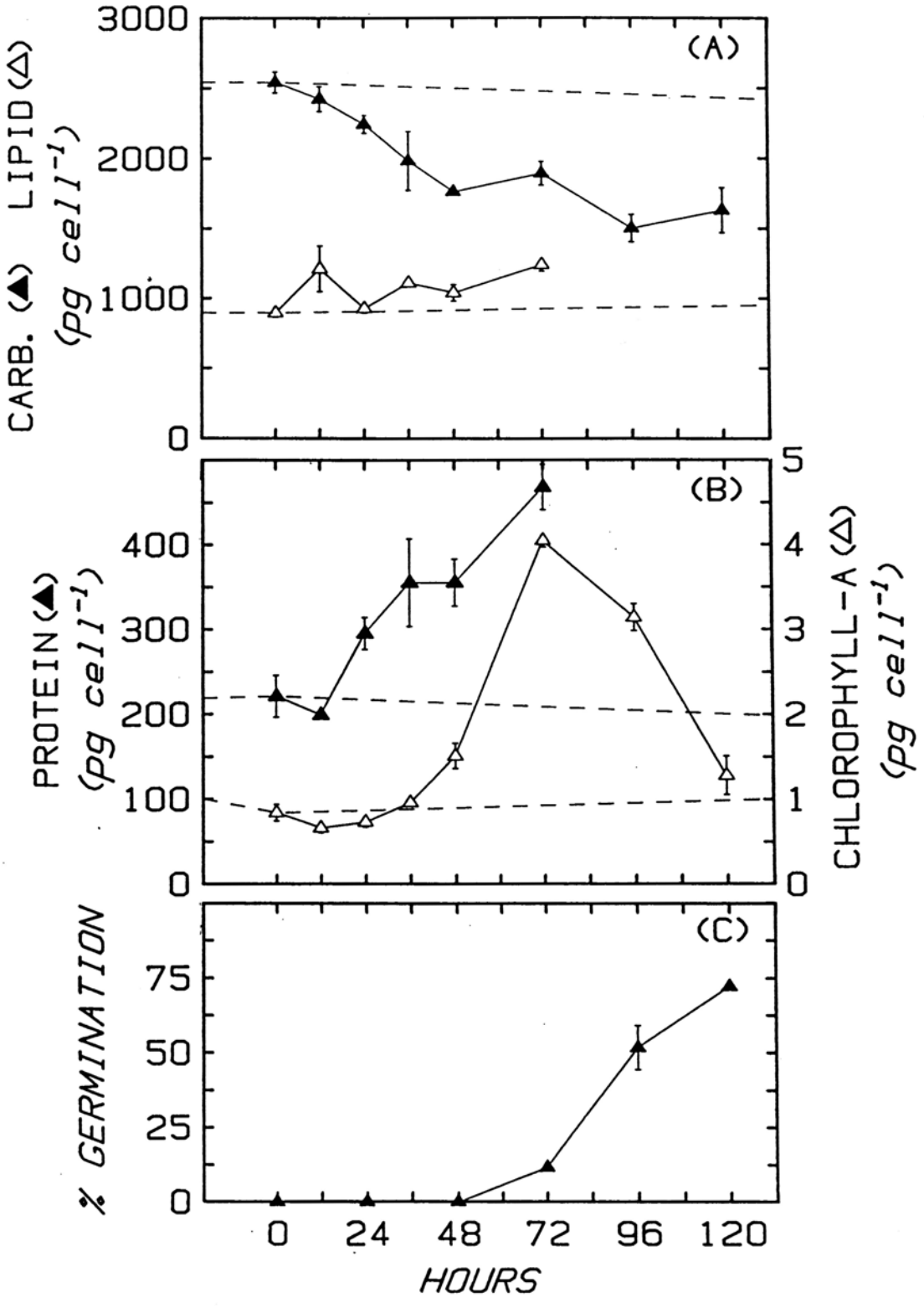


Fig. 2. (continued).

Fig. 3. Changes in cyst composition during germination. Cysts activated at time 0. A) Carbohydrate (▲) and lipid (△). B) Protein (▲) and chlorophyll-a (△). C) Germination frequency. Dashed lines indicate the trends in composition in quiescent cysts (Fig. 2). Means ±SE (n=3).


(A)
CARB. (▲) LIPID (△)
(pg cell⁻¹)
3000
2000
1000
0
(B)
PROTEIN (▲)
(pg cell⁻¹)
400
300
200
100
0
CHLOROPHYLL-A (△)
(pg cell⁻¹)
5
4
3
2
1
0
(C)
% GERMINATION
75
50
25
0
0
24
48
72
96
120
HOURS

70% of the cysts had germinated (Fig. 3C). The maximum germination frequency for this batch of cysts (measured just prior to the experiment) was 85%. Protein and lipid measurements do not extend beyond 72 hr because the decrease in sample size resulting from germination reduces these parameters to concentrations near or below their limits of detection.

The compositional changes observed in activated cysts were paralleled by changes in metabolic activity (Fig. 4). Cyst respiration rate increased by an order of magnitude during the first 3 days of incubation. Photosynthetic capacity ("P-gross") was unmeasureable initially and on day 1, but increased sharply thereafter. As a result of the large increase in respiration rate over the same period, however, net photosynthesis was not greater than zero until day 4, the first day that germination was observed. At that time the P-gross/R ratio reached approximately 1.4 (Table 2).

Despite the dramatic increases in respiration and photosynthetic capacity in activated cysts, the maximum observed rates were still considerably below the rates for exponentially growing S. trochoidea vegetative cells (Table 2). Thus, on a per cell basis, respiration rate and P-gross in fully activated cysts were approximately 50% and 30% respectively, of the values for vegetative cells. Note, however, that the estimated photosynthetic rate normalized to chl-a was reduced by only 25% in these activated cysts, relative to vegetative cells.

DISCUSSION

The data presented in this study afford for the first time a glimpse of the biochemical changes which accompany encystment, dormancy, and germination in dinoflagellates. They support the hypothesis that cysts and vegetative cells of S. trochoidea differ significantly in gross biochemical composition and metabolic activity. In particular, cysts contain large amounts of carbohydrate, which apparently provide the meager, though not insignificant, energy that quiescent cysts require. Respiration rate in quiescent cysts is reduced an estimated 60-fold, relative to healthy vegetative cells. Photosynthetic capacity was unmeasurable in quiescent cysts, although they did contain chlorophyll. These results are consistent with the common view that dinoflagellate cysts represent inactive life history stages equipped to survive extended periods under conditions unsuitable for vegetative growth.

Encystment. In considering the changes which accompany the transition from exponentially growing cells to resting cysts, it is important to distinguish between those changes which occur in vegetative cells in response to nutrient limitation and those which are specifically involved in cyst formation. As encystment in dinoflagellates is often induced by nutrient limitation (Pfiester and Anderson 1986), this distinction may seem somewhat arbitrary. However, the fact that 80% of the vegetative population does not encyst in my nutrient-limited experimental cultures (Chapt. 1) argues that the response to nutrient depletion and the process of encystment are indeed

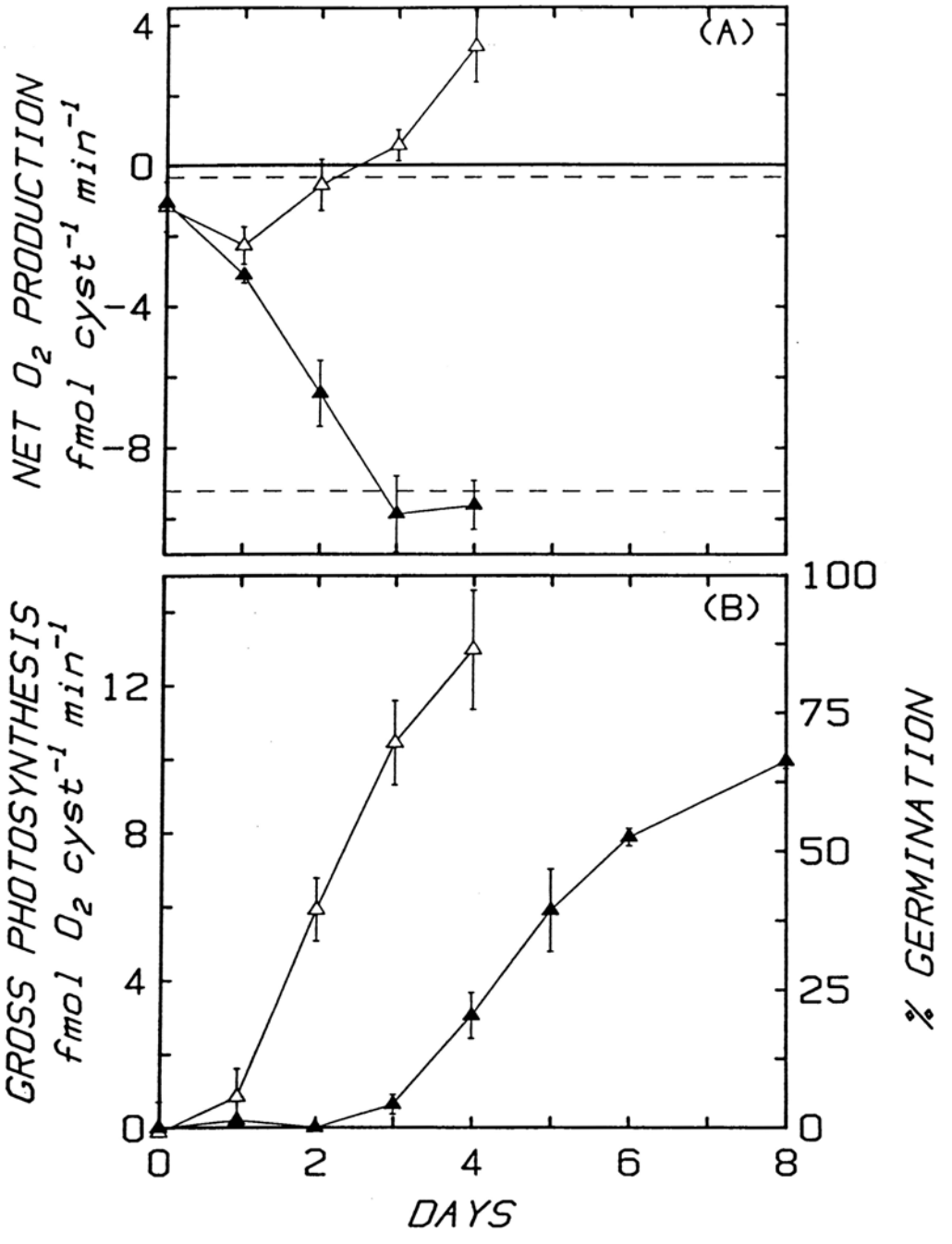


Fig. 4. Metabolic activity of cysts during germination. Cysts activated at time 0. A) Net oxygen production in the dark (▲) and under saturating light intensities (△). Broken lines indicate respiration rates of quiescent and germinating cysts calculated from carbohydrate loss (Figs. 2 and 3). Mean ±SE (n=2). B) Gross photosynthesis (△) calculated from A); bars indicate SE's (df=10; see Methods). (▲), germination frequency over time; mean ±SE (n=3).

separate, though perhaps not independent, phenomena.

The reduction in protein and chl-a and the increase in carbohydrate observed in vegetative S. trochoidea cells as they reached stationary phase are consistent with the changes generally observed in nitrogen-starved algal cultures (Fogg 1959, Myklestad 1974, Sakshaug and Holm-Hansen 1977, Morris 1981). Although lipid is accumulated in nitrogen-starved cells of some algae (Fogg 1959, Shifrin and Chisholm 1981), carbohydrate is clearly the predominant storage product in S. trochoidea; it increased approximately 500% in stationary-phase cells (on a per volume basis), while lipid increased only 40%.

The changes which accompanied cyst formation in S. trochoidea were qualitatively similar to those observed for nitrogen starvation in vegetative cells. In fact, if we assume that one cyst is formed by the sexual fusion of two stationary-phase cells (Watanabe et al. 1982), the protein, lipid, and chl-a contributed by both of those cells could account for the level of these constituents in cysts without any further synthesis or degradation (Fig. 1a, broken-line bars). Since the small population of vegetative cells which fuse and ultimately form cysts may not have the same composition as the "mean" stationary phase cell, the possibility of net synthesis or degradation of these constituents during encystment cannot be discounted at present.

In contrast to protein, lipid, and chl-a, carbohydrate was clearly accumulated in cysts at levels far exceding those in nitrogen-starved cells. The contribution of carbohydrate to a cyst by a pair of stationary-phase cells would account for less than a third of the total cyst carbohydrate content. Thus, active carbohydrate synthesis and

accumulation represents a major biochemical change specifically accompanying encystment. This finding is consistent with the common observation of increases in starch granules in various dinoflagellate cysts, relative to vegetative cells (Wall and Dale 1969, von Stosch 1973, Anderson 1980, Chapman et al. 1982). The time period during which the synthesis of carbohydrate occurs cannot be descerned with the present data. However, microscopic studies of dinoflagellate encystment suggest that the planozygote stage may be responsible for the accumulation of these storage products (von Stosch 1973, Chapman et al. 1982). This conclusion is supported by the demonstration of active photosynthesis in G. tamarensis planozygotes (Anderson unpubl.).

The high carbohydrate/lipid ratio in cysts (3.0) relative to that in exponential-phase and stationary-phase vegetative cells (0.3 and 1.1, respectively) emphasizes the increased importance of carbohydrate as a storage compound in cysts. Still, due to the high theoretical ATP-yield of lipid compared to carbohydrate (504 mol/kg vs. 206 mol/kg), the "energy" stored in cyst carbohydrate is only marginally greater than that in lipid (these calculations assume that all carbohydrate and all lipid is available for respiration). Note that carbohydrate, rather than lipid, is a particularly appropriate storage product in an organism which is likely to encounter anoxic conditions (Hochachka 1980).

Although no dramatic accumulation of lipid was apparent in S. trochoidea cysts (compare the per-volume lipid content in exponential cells and cysts, Fig. 1b), this does not preclude changes in relative lipid class composition, as has been observed in other algal resting

Table 2. Comparison of the respiratory and photosynthetic activity in cysts and vegetative cells. Data for activated cysts from Fig. 4. Values in parentheses not significantly different than zero, but included for comparison. Numbers in brackets refer to the degrees of freedom associated with the standard error estimate (see Methods).

		fmol O_2 $cell^{-1}$ min^{-1} Mean ±SE [df]	nmol O_2 μg Chl-a^{-1} min^{-1} Mean ±SE	P/R ±SE
RESPIRATION				
Cysts				
Quiescent	(3°C)	0.34 ±0.034[1] [3]	NA	NA
Activated (day 0)	(18°C)	(1.1 ±0.59) [10]	(1.4 ±0.79)[2]	(-0.12 ±0.79)
(day 4)	(18°C)	9.6 ±1.16 [10]	2.4 ±0.28[2]	1.35 ±0.234
Vegetative Cells				
	(18°C)	22.8 ±1.95 [7]	1.9 ±0.16	2.20 ±0.220
GROSS PHOTOSYNTHESIS				
Cysts				
Activated (day 0)	(18°C)	(-0.12 ±0.83) [10]	(-0.17 ±1.1)[2]	
(day 4)	(18°C)	13.0 ±1.63 [10]	3.21 ±0.40[2]	
Vegetative Cells				
	(18°C)	50.3 ±2.58 [7]	4.12 ±0.21	

[1]based on loss of carbohydrate (see Methods)
[2]based on day 0 or day 3 chl-a $cyst^{-1}$ values in Fig. 3

stages (Berkaloff and Kader 1975, Lichtlé and Dubacq 1984). In particular, the decrease in membrabnous structures and increase in lipid droplets observed in the dinoflagellate Woloszynskia tylota during encystment (Bibby and Dodge 1972) could have reflected an increase in triacylglycerides (storage lipids) at the expense of phospholipids and galactolipids (membrane components).

Dormancy and quiescence. Scrippsiella trochoidea cysts undergo a period of dormancy lasting approximately 25 days, during which germination does not occur (Chapt. 1). After this period, cysts incubated under permissive conditions germinate, while those kept under non-permissive conditions remain quiescent. It is appropriate, therefore, to separately consider the changes which occur during the dormancy period (between day 21 and day 49 in the present study) and those which occur subsequently, among quiescent cysts.

The loss of both carbohydrate and lipid between days 21 and 49, at both 3°C and 18°C, suggests significant metabolic activity during the dormancy period in S. trochoidea cysts (Fig. 2, broken lines). The rate of carbohydrate loss at both temperatures in this time interval was comparable to the long term rate of loss in 3°C cysts, and greater than the long term rate at 18°C (see below). Lipid degradation was significantly greater at 18°C than at 3°C during dormancy, presumably reflecting a higher rate of respiration at that higher temperature. Although variability in the data makes quantitation of lipid disappearance tenuous, the calculated ATP contribution from lipid during dormancy is comparable to that from carbohydrate in cysts stored at 3°C, and is 3 times that of carbohydrate for cysts at 18°C. The

estimated overall respiration rate at 18°C (2.5 fmol O_2 $cyst^{-1}$ min^{-1}) is still only 10% of the rate measured in vegetative cells (Table 2).

The patterns of carbohydrate and lipid utilization established during dormancy were greatly altered as cysts entered the quiescent state. Although the rate of loss of carbohydrate remained approximately constant in 3°C cysts over the entire experiment, it was greatly reduced in 18°C cysts after day 49. Thus, the calculated respiration rate was 4 times greater in cysts stored at 3°C than in those stored at 18°C. The explanation for this difference is not clear. Respiration rate is expected a priori to decrease or at best (in the case of some eurythermic algae) to remain constant as temperatures decrease (Ryther and Guillard 1962, Soeder and Stengel 1974). It is conceivable that respiration in S. trochoidea cysts could be regulated so that it increases at lower temperatures. This might reflect a higher respiratory efficiency in cysts stored at higher temperatures, or perhaps a higher maintainance cost at lower temperatures. More experiments are obviously required before the influence of temperature on respiration in quiescent cysts can be firmly established.

If the rate of carbohydrate loss in quiescent cysts remains constant over time, cysts stored at 3°C should consume all of their carbohydrate reserves in 240 days. The observation of sustained viability in S. trochoidea cysts for at least 350 days (Chapt. 1) therefore suggests that either respiration rate decreases as cysts age, or that lipid or protein become important as respiratory substrates.

The gradual decrease in carbohydrate in quiescent cysts was accompanied by a gradual increase in lipid. Although the variability in the lipid data is high with respect to the changes observed, statistical analysis (Table 2) suggests that the increase in lipid over time is real. Again, these results are unexpected and difficult to explain. That lipid can be synthesized at the expense of endogenous reserves in the dark is not surprising (Miller 1962). However, such synthesis is quite expensive in terms of ATP and reducing power (Lehninger 1975), and its possible benefit to quiescent cysts is not immediately apparent. Interestingly, net synthesis of lipids in diatom resting cells at low temperature in the dark was reported by Anderson (1975). Furthermore French and Hargraves (1980) observed an increase in cellular carbon in diatom resting spores stored in the dark. These observations imply the presence of a significant anabolic component in the overall metabolism of resting cells which may be reflected in the increased lipid content of S. trochoidea cysts as well.

The lack of measureable photosynthetic activity immediately subsequent to activation (Fig. 4) indicates that quiescent cysts retain very little, if any, photosynthetic capacity, despite the presence of chl-a. Had the photosynthetic rate per chl-a been equal to that in vegetative cells, gross photosynthesis should have been detectable on day 0. Therefore the reduced photosynthetic capacity among quiescent cysts appears to result from a disruption of the cellular photosynthetic machinery beyond a simple reduction in chl-a content.

Overall, these data confirm the common assumption of reduced metabolic activity in dinoflagellate cysts. The highest estimate of

respiratory activity among non-activated cysts was observed during the dormancy period in 18°C-stored cysts and was an order of magnitude below the respiration rate measured in vegetative cells. The estimate of respiration rate among quiescent cysts is even lower, at approximately 1.5% of the vegetative rate (Table 2).

The loss of membranous cytoplasmic components and disruption of chloroplasts observed by Bibby and Dodge (1972) during W. tylota encystment most probably reflect reductions in respiratory and photosynthetic capacity analagous to that observed in S. trochoidea. The apparent elevation of metabolic activity in young (dormant) cysts relative to older (quiescent) cysts in S. trochoidea supports the suggestion that the mandatory dormancy period observed in many dinoflagellate cysts may not represent a period of "rest" as much as it does a period of development (Dale 1983). This observation is consistent with the relatively rapid disappearance of storage products observed in Gonyaulax tamarensis cysts during the first few weeks of storage (Anderson 1980).

Germination. Not surprisingly, increased metabolic activity accompanied germination in S. trochoidea cysts (Figs. 3 & 4, Table 2). The respiration rate calculated from carbohydrate loss in activated cysts is comparable to that measured directly, although differences in germination kinetics make direct day to day comparisons impossible. The stimulation of respiratory activity began within 12 hr of activation, and thus preceded protein synthesis and the increase in chl-a and photosynthetic activity. The relationship between these events cannot be established with the data at hand, but it seems

reasonable that nitrogen uptake and protein synthesis would be dependent upon energy derived through respiratory metabolism, and that the increase in chl-a and overall re-activation of photosynthesis would in turn require newly synthesized enzymes and photosynthetic proteins (Fig. 5). The drop in chl-a content observed at 96 and 120 hr (at which point more than 50% of the cysts had germinated) most likely reflects the composition of those cysts which hadn't yet germinated, rather than a net decrease of chl-a within these cysts over time. Thus it appears that those cysts which did not germinate (or germinated slowly) were those which were low in chl-a content. The causal relationship between these two variables cannot be established at present.

The overall dependence of germination on endogenous energy reserves in S. trochoidea cysts is indicated by the relatively low P/R ratios observed in activated cysts (Fig. 4, 5). These range from essentially zero on day 0 to 1.4 just prior to germination. The general correspondence between carbohydrate disappearance and measured oxygen consumption suggests that carbohydrate acts as the major respiratory substrate during this period. This conclusion is supported by the lack of significant changes in the lipid content of cysts over the same interval.

Although respiratory and photosynthetic activity increase dramatically prior to excystment, the fact that both are still below the rates measured in vegetative cells (Table 2) argues that the full restoration of vegetative metabolism in these cells is not complete until after excystment. The extent to which the observed changes in

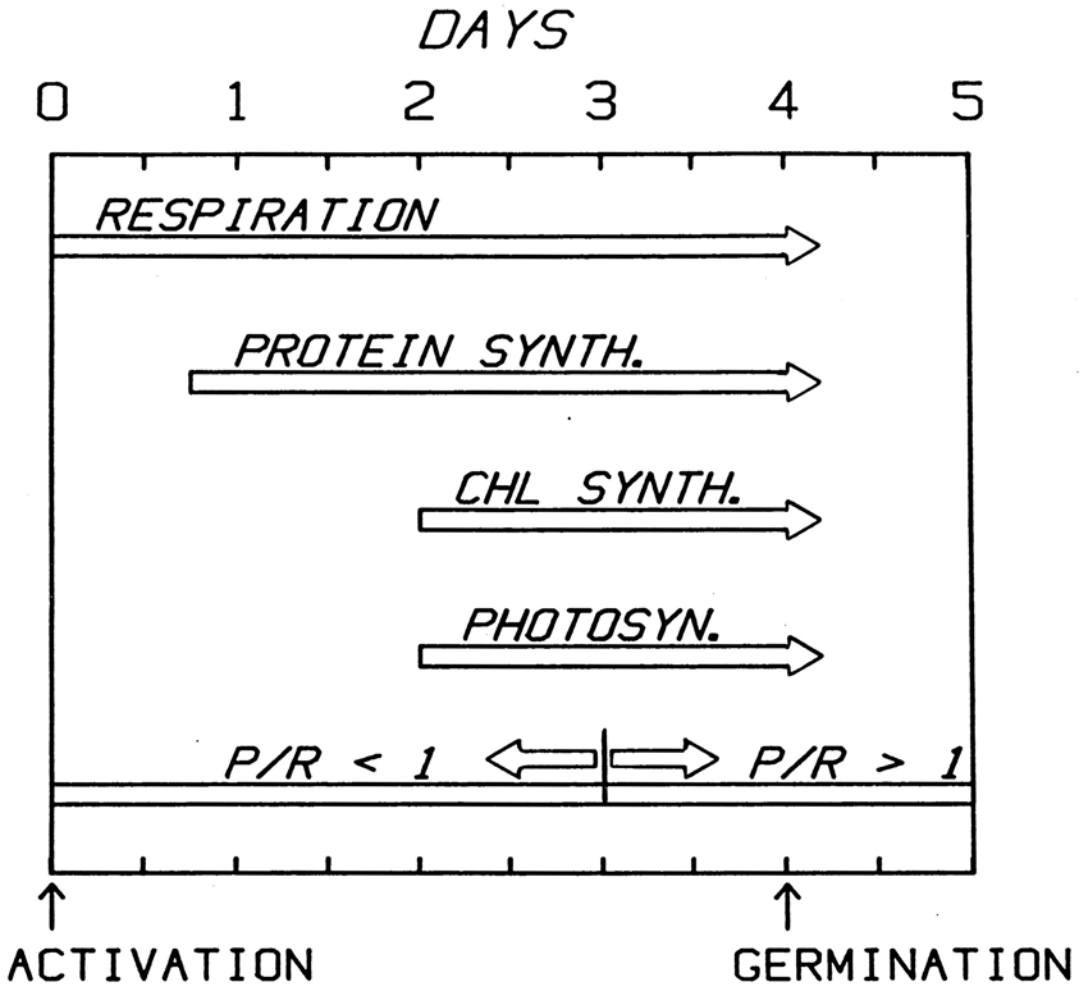


Fig. 5. Summary of the metabolic events hypothetically leading to germination in S. trochoidea cysts. See text for discussion.

activity are prerequisites for germination remains uncertain. The ability of S. trochoidea cysts to germinate in the dark after only brief exposure to light (Chapt. 2) demonstrates that photosyntheic activity is not required for germination. Likewise, germination in nitrogen-poor medium (Chapt. 1) suggests that protein synthesis is not a requirement, although the role of endogenous compounds in providing the necessary nitrogen for such synthesis cannot be discounted. Yet in both cases, germination rate and/or frequency is often improved by the addition of light or a nitrogen source, respectively. Thus, while neither photosynthesis nor protein synthesis appears to be an absolute requirement for germination, both can influence its rate and success.

The extent to which respiratory activity is a prerequisite for germination is not known. Reports of germination by Ciratium hirundinella cysts under anoxic or hypoxic conditions (Huber and Nipkow 1923, Krupa 1981) suggest that such activity is not required for germination in this species. In contrast, the apparent lack of anoxic germination in cysts of other dinoflagellates (Endo and Nagata 1984, Anderson in prep.) suggests that aerobic respiration could be a prerequisite for germination in many species.

Comparisons with other algal resting stages. Resting stages are known among members of most algal classes (Fryxell 1983). Those for which the most physiological data are available include cyanophyte akinetes (Nichols and Carr 1977), diatom resting cells and spores (Hargraves and French 1983), and chlorophyte akinetes (Coleman 1983).

The accumulation of storage products, as observed here in S. trochoidea cysts, occurs in all of these resting forms, and in fact in

dormant stages of a wide variety of oganisms (Sussman and Douthit 1973, Bewley and Black 1978). In cyanophyte akinetes, glycogen and cyanophycin (composed of polypeptides) serve as storage products (Wildman et al. 1975, Sutherland et al. 1979), while lipid and/or starch are accumulated in the resting cells of diatoms, chlorophytes, and cryptophytes (Anderson 1975, Berkaloff and Kadar 1975, Lichtlé 1979, Doucette and Fryxell 1983, O'Neal and Lembi 1983).

Although reduced metabolic activity is another characteristic common among dormant stages (Sussman and Halvorson 1966, Sussman and Douthit 1973, Bewley and Black 1982), the metabolic profile of algal resting stages is quite variable. Thus, while decreases in Nostoc akinete respiration were comparable to those reported here in S. trochoidea cysts (Chauvat et al. 1982), respiration rate in the akinetes of another cyanophyte, Anabaena cylindrica, were actually greater than those in vegetative cells (Fay 1969a, Yamamoto 1976). Likewise, respiration rates in Pithophora oedogonia (Chlorophyceae) akinetes were greater than or equal to vegetative rates (O'Neal and Lembi 1983). Respiration in diatom resting stages is generally less than that in vegetative cells (Anderson 1976, French and Hargraves 1980).

Generalizations concerning resting stage photosynthetic capacity are equally difficult to make. Although reductions in photosynthetic capacity similar to that observed here in S. trochoidea cysts are evident in chlorophyte and cyanophyte akinetes, and in some diatom resting cells (Fay 1969a, Yamamoto 1976, Chauvat et al. 1982, O'Neal and Lembi 1983), photosynthesis in other diatom spores is apparently

comparable to that in vegetative cells (French and Hargraves 1980, Hollibaugh et al. 1981). Despite their reduced photosynthetic capacity, cyanophyte akinetes contain chl-a at levels comparable to vegetative cells (Fay 1969b, Sutherland et al. 1979, Chauvat et al. 1982). Not surprisingly, diatom resting spores and vegetative cells also have similar chl-a contents (Anderson 1975, French and Hargraves 1980, Doucette and Fryxell 1983).

Differences in resting stage metabolic capacities notwithstanding, the general pattern of metabolic activity during germination in many algal resting stages is similar to that observed here in S. trochoidea cysts (Fig. 5). In particular, an early increase in respiratory activity, a dependence on respiration-derived energy during initial stages, and a delayed but marked increase in photosynthetic activity characterize germination in many algae (Hommersand and Thimann 1965, Chauvat et al. 1982, O'Neal and Lembi 1983). As suggested above for S. trochoidea, this series of observed events may reflect a developmental program involving a stimulation of respiration, the use of the resulting energy in RNA and protein synthesis, and finally the application of newly synthesized enzymes and protein components to the reconstitution of the photosynthetic system. Chauvat et al. (1982) presented evidence for just such a cascade of events in germinating Nostoc akinetes.

The germination of Chaetoceros resting spores provides a conspicuous exception to this paradigm; spores of this species begin to photosynthesize at relatively high rates immediately upon exposure to light, even after 167 days of cold dark storage (Hollibaugh et al.

1981). Interestingly, these diatom resting spores appear to require photosynthesis for germination, while chlorophyte akinetes and dinoflagellate cysts do not (Huber and Nipkow 1923, Neal and Herndon 1968, Anderson and Wall 1978, Anderson in prep). On the other hand, there is good evidence that photosynthesis is a requirement for germination in Nostoc akinetes, even though increased photosynthetic activity lags behind the increase in respiration (Chauvat et al. 1982).

In overview, cysts of Scrippsiella trochoidea appear to represent resting stages as "restful" as any observed among the algae. The extent of reduction in respiratory and photosynthetic capacity they display, combined with their significant accumulation of storage compounds, is consistent with their often presumed, but rarely tested, role as dormant perreniating cells.

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Appendix I

Evidence for Bacterial Inhibition of Cyst Production in *Scrippsiella trochoidea*

The environmental factors that induce sexuality and encystment in dinoflagellates are not well understood. Although nutrient limitation is often implicated, the specific events which are involved in sexual induction and cyst formation, the timing of these events, and the mechanisms by which these events are regulated remain problematic (Anderson et al. 1985, Pfiester and Anderson 1986).

In this appendix, evidence for microbially mediated inhibition of cyst production in Scrippsiella trochoidea is presented. Identification of the specific factors responsible for this inhibition should add to our understanding of the regulation of dinoflagellate sexuality and encystment.

In order to establish axenic clonal cultures of S. trochoidea, single cells were isolated by micro-pipette under aseptic conditions from a ScrpMxB culture (Chapt. 1). These cells were repeatedly micro-pipetted into drops of sterile medium and finally transferred to culture tubes and incubated under standard conditions (Chapt. 1). Nine clonal cultures were established in this way.

As an initial test of whether sexual reproduction in S. trochoidea is homothallic or heterothallic, these clones were innoculated singly and in pairs into standard encystment medium (Chapt. 1). Approximately 1 week after the in vivo fluorescence of the cultures had peaked, cyst concentrations were determined.

No difference in cyst yield was noted among clones or between self-crossed and inter-crossed cultures, except that cyst production by clone SA2 was abnormally low (<1% of the mean yield among other

Table 1. Cyst yield of clones (diagonal axis) and selected crosses of clones. Cysts produced per ml of culture; mean of two replicate cultures per cross. Average CV within crosses was 25%.

Clone → ↓	SA1	SA5	SA9	SA2	SA3	SA6	SA7	SA8	SA10
SA1	1050	900	755	2	1450	970	960	780	1470
SA5		1060	620	4	1010	940	820	750	1090
SA9			700	4	1010	560	910	780	530
SA2				5					
SA3					1230				
SA6						900			
SA7							400		
SA8								1130	
SA10									1220

clones) (Table 1). Furthermore, in any two-clone crosses involving SA2, cyst production was likewise reduced. This reduction in cyst yield was not the result of reduced vegetative cell growth; final vegetative cell numbers were in fact slightly higher in the crosses involving SA2 (Fig. 1). Neither was the reduction merely a reflection of delayed cyst formation; examination of cultures 2 months later revealed no significant increase in cyst number.

The observation that among the nine clones tested SA2 alone proved positive for bacterial contamination (as judged by growth in test broth: f/2 + glucose, bacto-tryptone, and yeast extract, each at 0.1%) led to the hypothesis that a bacterium was responsible for the reduction in cyst yield observed in that clone.

To test this hypothesis, sub-clones were established from SA2 using the methods described above. One such sub-clone, designated SA2(1), tested negative for bacterial growth in test broth, and produced cysts at levels comparable to the originally isolated non-contaminated clones (Fig. 2). Crosses between SA2(1) and SA1 (a non-contaminated clone) resulted in good cyst production, while crosses between either of these clones and SA2 resulted in poor cyst yield.

Introduction of 200 µl of an 8 µm (Nucleopore) filtrate from a stationary-phase SA2 culture at the start of the these incubations inhibited cyst formation to the same extent as introduction of SA2 itself (Fig. 2). This filter pore size prevented passage of S. trochoidea cells (as confirmed by microscopic examination), but should have allowed passage of bacterial cells.

Although these data strongly suggested that the inhibition of S.

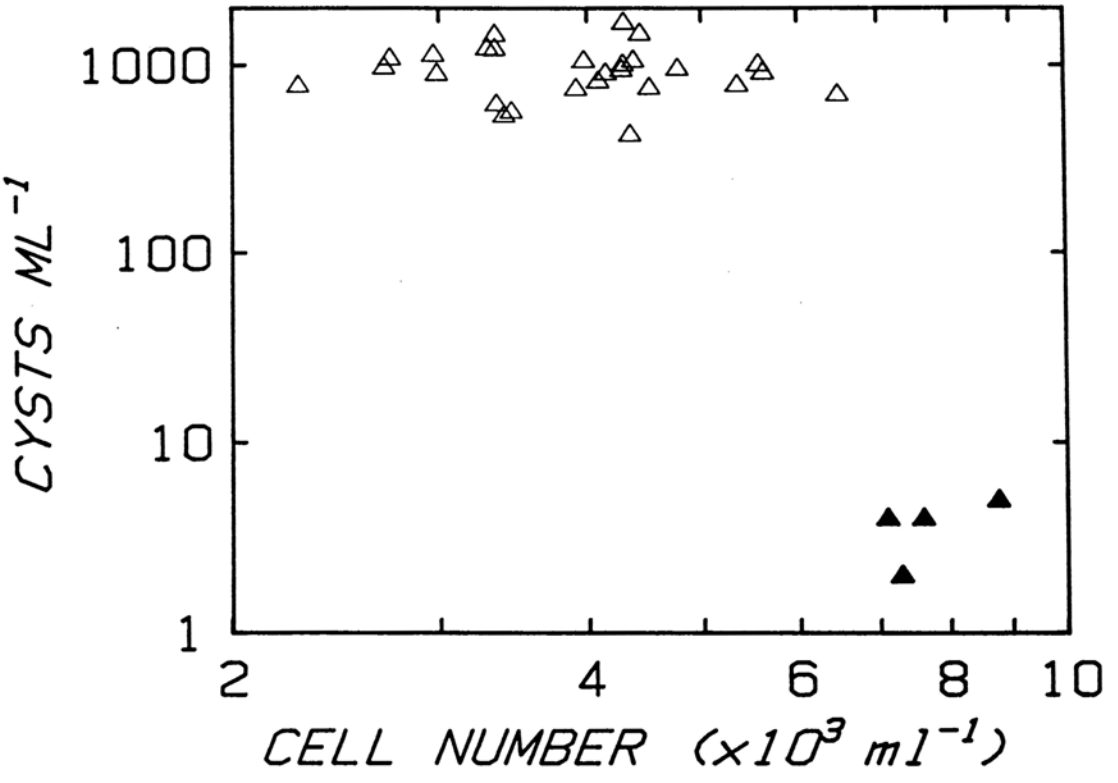

Fig. 1. Relationship between cyst yield and vegetative cell yield in clonal and intercrossed cultures (log-log plot). Specific cultures are listed in Table 1. Closed symbols are crosses involving clone SA2; open symbols are all others. Means of two replicate cultures of each cross are shown.

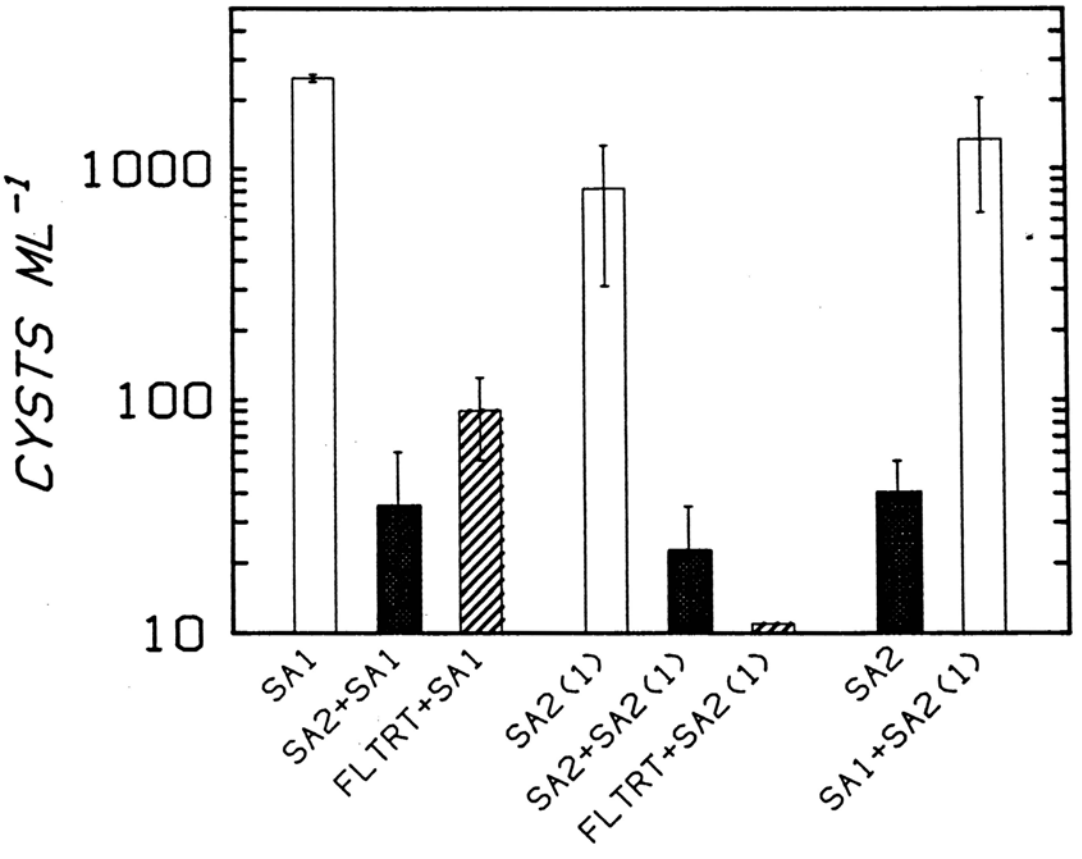

Fig. 2. Cyst yield in SA1 (an axenic clone), SA2 (the contaminated clone), and SA2(1) (an axenic sub-clone of SA2). Note the log scale. "Fltrt" refers to an 8 μm filtrate of SA2 (see text). Closed bars denote cultures receiving SA2; hatched bars denote cultures receiving SA2 filtrate. Error bars show the range of cyst yields in two replicates of each treatment.

trochoidea encystment was microbially mediated, the final proof of this hypothesis was not forthcoming. I could identify three morphologically distinct colony types from streaks of SA2 on marine agar medium (0.1% glucose and bacto-tryptone, 0.05% NH_4NO_3, 0.01% yeast extract, and 2.2% agar in seawater), but none of these bacterial isolates proved effective in reducing cyst yield when introduced into SA1 cultures (Fig. 3). Therefore, the conclusion that a bacterial contaminant in SA2 was responsible for the observed inhibition of encystment must remain tentative.

The mechanism by which a bacterium might exert this effect is not clear. The good vegetative growth exhibited by SA2, combined with the ease with which it was freed from its contaminants argues against direct pathological involvement. A preliminary experiment showed that a 0.2 μm filtrate from SA2 was effective in reducing encystment in axenic clones, suggesting the production of an inhibitory compound. However failure to demonstrate that the filtrate was in fact free of bacteria in this experiment makes unequivocal interpretation of the data difficult.

In light of the widely reported importance of nutrient depletion for the induction of sexuality in dinoflagellates, a bacterially mediated change in nutrient concentration or dynamics is a plausable explanation for the observed inhibition. However the fact that this inhibition appears to occur in the presence of only certain bacteria argues against a general nutrient regeneration effect, and in favor of some specific change, mediated by a specific bacterium.

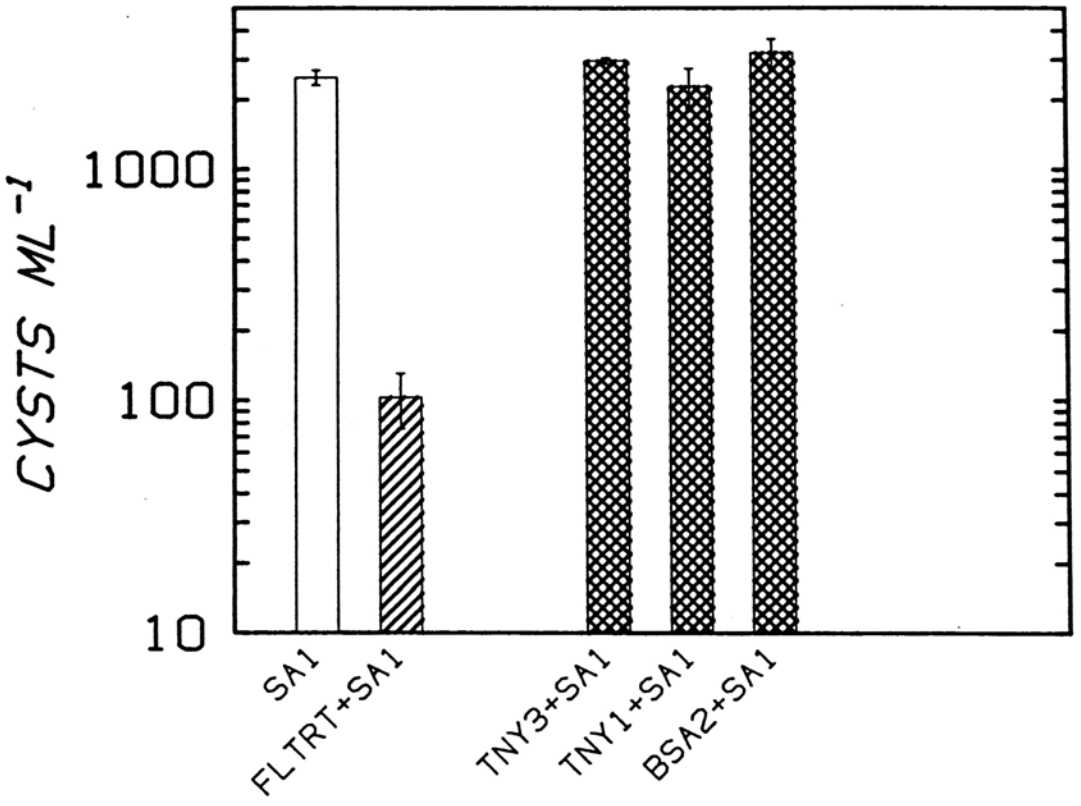

Fig. 3. Effect of bacterial isolates on cyst yield in SA1 cultures. Note log scale. "TNY3," "TNY1," and "BSA2" refer to specific bacterial isolates from SA2. Cross-hatched bars denote cultures innoculated with these isolates; hatched bar is negative control, receiving SA2 filtrate (as in Fig. 2); open bar is positive SA1 control. Data and error bars as in Fig. 2.

The question remains open. Future studies which identify the factor(s) responsible for the inhibition of cyst formation in S. trochoidea clone SA2 would certainly contribute to our knowledge of the environmental and biological regulation of dinoflagellate sexuality and encystment, and might provide a useful tool for further investigation of these phenomena.

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Appendix II

Notes on Methods

The methods employed in this thesis are in most cases detailed within Chapters 1-3. The rational behind those methods, and some additional details, are presented in this appendix.

Sequential extraction. Prior to biochemical analyses, samples were sequentially extracted to yield "lipid," "carbohydrate," and "protein" fractions. The details of the extraction employed are given in Chapt. 3; the scheme is outlined in Table 1 of this appendix.

The primary reason for employing a sequential extraction in the present study was the minimization of the total amount of biomass necessary for the desired assays. If conservation of experimental material were not a consideration, direct analysis of separate, unextracted samples would be a reasonable alternate strategy. Note, however, that pre-extraction has the additional advantages of eliminating compounds which might interfere with the assays employed, and in certain cases of actually increasing the yield of a particular assay (e.g. see Protein, below).

The disadvantages of sequential extraction include increased analytical complexity and the potential loss of constituents to other fractions. These problems are discussed in detail in Roberts et al. 1955, Sutherland and Wilkinson 1971, and Hitchcock 1983. Note that one of the major problems of such extraction schemes, particularly as applied to dinoflagellates, is the insolubility of cell wall constituents (e.g. cellulose) in the standard solvents, and the resultant carry-over of these constituents into the final "protein" fraction (Hitchcock 1983). Since I assayed protein and carbohydrate

Table 1. Outline of sequential extraction scheme employed in the present study. CM = 2:1 chloroform/methanol; (s) = soluble fraction; (i) = insoluble fraction. Centrifugation at 2000 xg for 20 min was used to separate (s) and (i) at each extraction step. See Chapt. 3 (Methods) for details.

1) Store sample (on glass fiber filter) in CM at -20°C		
2) Grind filter and sample		
3) CM Extraction	(s)→	LIPID & CHLOROPHYLL
↓(i)		
4) CM Extraction	(s)→	
↓(i)		
5) TCA Extraction (5%, 90°C, 20 min)	(s)→	CARBOHYDRATE
↓(i)		
6) NaOH Extraction (0.1 N, 90°C, 20 min)	(s)→	PROTEIN (& CARBOHYDRATE)

chemically, the appearance of such non-protein contaminants in the "protein" fraction is not of consequence in the present study.

Hitchcock (1983) presented a detailed comparison of extraction schemes as applied specifically to dinoflagellates. He found that the methods of Li et al. (1980) and Kochert (1978) yielded comparable results. The extraction employed in the present study is based on the former method. The major modifications of this method include 1) storage of samples in 2:1 chloroform-methanol ("CM") at -20°C under N_2, 2) grinding of filter and sample rather than reliance upon freezing and thawing for disruption of cells, and 3) separation of particulate and soluble phases at each step via centrifugation rather than filtration. The first modification was made because such storage conditions are preferable for the preservation of lipids and proteins. The second seemed advisable in light of the probable resistance of cysts to breakage. The third simplified the required manipulations, although it also increased the time required for these manipulations considerably.

The efficacy of particular extraction steps is addressed in the sections below.

Protein. The Coomassie Brilliant Blue dye-binding assay I employed for protein analysis is described in Chapt. 3. The assay was chosen on the basis of its sensitivity and simplicity (Bradford 1976, Spector 1978). I found, however, that "micro" versions of the assay (like the one employed here) were quite sensitive to small changes in the chemistry of the assay mixture. For this reason, standards were prepared in blank solutions whose chemistry was identical to the

sample solutions in question (see Chapt. 3).

Solubilization of protein under hot alkali conditions preceded the dye-binding assay. The specific conditions employed for such solubilization in algae have varied widely (see Rausch 1981). Generally, a balance must be struck between the need for efficient extraction on one hand, and the desire to minimize protein denaturation on the other (Rausch 1981). In order to optimize extraction conditions in the present study I tested the effect of extraction time and NaOH concentration on protein yield from vegetative S. trochoidea cells (Fig. 1, Table 2). Extraction temperature was set at 90°C, as recommended by the results of Rausch (1981). On the basis of these data, an extraction time of 20 min, and an NaOH concentration of 0.1 N was adopted as the standard conditions for protein solubilization.

The liberation of protein from vegetative S. trochoidea cells was increased by a factor of 2 by pre-extraction with hot TCA (not shown). This is presumably the result of the breakdown by TCA of barriers which otherwise limit the availability of solvent to protein (Hitchcock 1983). In contrast, pre-extraction in CM resulted in an estimated 20% loss of protein, presumably reflecting a soluble protein fraction. This loss was not corrected for in the protein data reported.

Protein standards, like samples, are suseptable to denaturation under extraction conditions. I found that the response of the assay to Bovine Plasma Gamma Globulin ("BPGG") dropped as extraction time or NaOH concentration increased. Control assays of BPGG standards in "extracted" blank solutions demonstrated that the change in response was due to changes in the protein and not to changes in the assay-

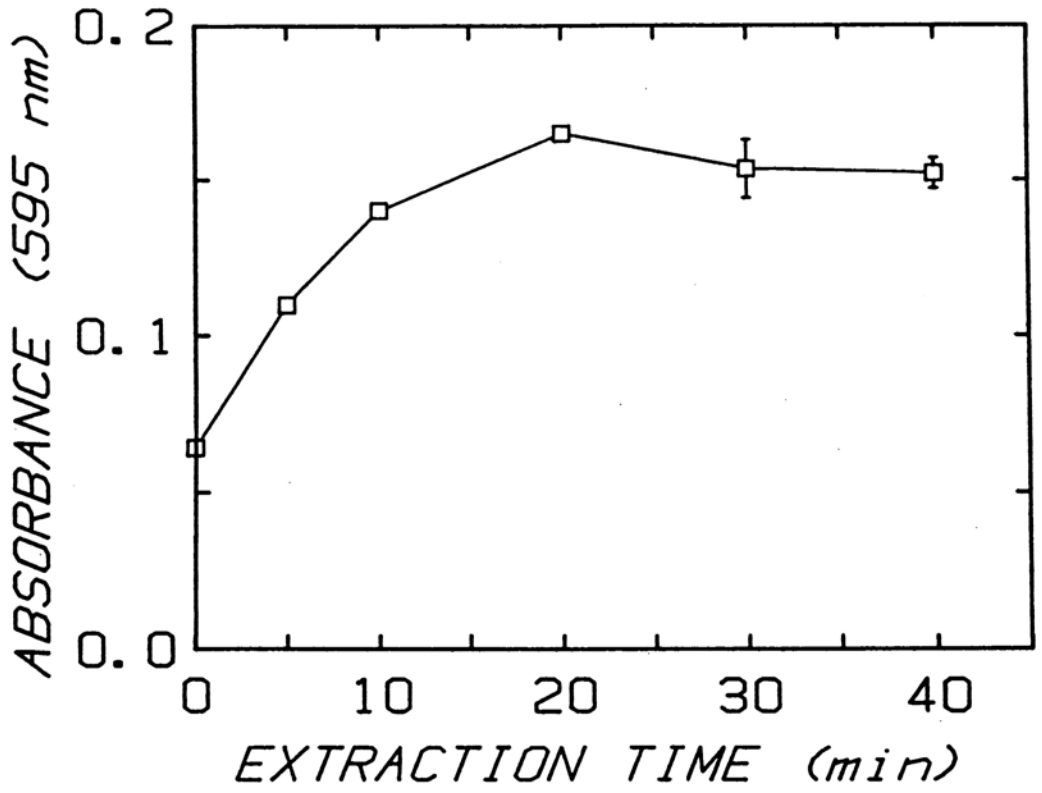

Fig. 1. Effect of extraction time on protein yield from vegetative S. trochoidea cells. Extraction temperature is 90°C; NaOH concentration is 0.1 N. Cells received no pre-extraction. Shown is the absorbance generated by the protein dye-binding assay (corrected for blank). Means ± SE (n=2).

Table 2. Comparison of protein yield from vegetative S. trochoidea cells extracted at 90°C for 10 min in different NaOH concentrations. Cells were pre-extracted with CM and hot TCA, per usual. Standards are undigested BPGG in digested blank solution (see text).

NaOH Normality	pg Protein $cell^{-1}$ Mean ± SE (n=3)	Standard Curve Slope Abs • $(\mu g\ ml^{-1})^{-1}$
1.0 N	490 ± 55	2.87×10^{-3}
0.5 N	590 ± 16	2.82×10^{-3}
0.1 N	1640 ± 74	2.78×10^{-3}

mixture chemistry. Therefore, non-digested protein standards diluted in digested blank solutions were employed as standards in all protein assays. The estimated limit of detection for this assay, under the conditions outlined above, was 0.5 µg protein ml^{-1} sample solution.

Lipid and Chlorophyll-a. The Bligh and Dyer (1959) lipid extraction is well established, and was not modified to any great extent for the present study. $MgCl_2$ was added to the CM fraction prior to this extraction in accordance with the recommendation of Folch et al. (1957). The double extraction employed (Chapt. 3) reduced the chance of non-lipid contamination and appeared to reduce the lipid blank as well. The blank which remained (~6 µg in 500 µl) is probably the result of inefficient pelleting of the sample and ground filter through the dense chloroform layer. However increased centrifuge speeds or times were not effective in reducing the blank. The estimated limit of detection for lipid in the present extraction scheme was 20 µg ml^{-1} final suspension.

The acidification of the CM fraction for quantification of pheopigments did not affect the subsequent recovery of lipid from vegetative S. trochoidea cells. Extraction of chlorophyll-a from vegetative cells with CM and with 90% acetone was identical, as judged fluorometrically (data not shown).

Carbohydrate. The carbohydrate analysis of Dubois et al. (1956) was employed without significant modification (Chapt. 3). By reading absorbances in the reaction tubes themselves, the need for transferring the viscous, corrosive assay mixture is avoided. I found that the

variability introduced by optical defects in the disposable tubes was insignificant.

Under the present extraction scheme, monosacharides and other soluble sugars are contained in the aqueous (non-lipid) fraction of the Bligh and Dyer lipid separation. In vegetative S. trochoidea cells, such sugars account for approximately 5% of the total carbohydrate (Table 3); they have not been measured in cysts. Table 3 also shows that in this case, 80% of the total cellular carbohydrate is effectively extracted in hot TCA, while 14% remains in the TCA-extracted pellet. In contrast, Hitchcock (1983) found that a large proportion of cellular carbohydrate in Gonyaulax tamarensis was hot TCA insoluble. The basis for this difference is not yet clear. Carbohydrate was measured in both the TCA and the final NaOH fractions throughout the present study, and in no case did the proportion of carbohydrate in this second fraction exceed 35% for vegetative cells or 22% for cysts. Changes in this proportion were not obviously correlated with any particular experimantal factor; the carbohydrate values reported in Chapt. 3 represent the sum of the TCA and NaOH carbohydrate measurements.

Glucose was employed as the standard for carbohydrate measurements; the response to soluble starch was essentially identical. Unlike the Bradford protein assay, the carbohydrate assay was comfortably robust with respect to assay-mixture chemistry. Still, for the reasons mentioned in the Protein section, undigested glucose solutions diluted in digested blank were employed as standards.

Respiration and Photosynthesis. Perhaps the largest concern with

Table 3. Distribution of carbohydrate from vegetative S. trochoidea cells among the Low Molecular Weight, TCA, and NaOH fractions. Extractions as described in Chapt. 3. Data presented as total carbohydrate in the cell suspension.

Fraction	µg Carbohydrate Mean ± SE (n=5)	% Total
LMW[1]	3.6 ± 0.79	5.3
TCA	54.7 ± 1.6	80.4
NaOH	9.6 ± 0.29	14.1
TOTAL	68.0	

[1]LMW = Low Molecular Weight fraction; the aqueous (non-lipid) fraction from the Bligh and Dyer separation.

regard to oxygen electrode measurements on dinoflagellates is the adverse effect of vigorous stirring on these sensitive organisms. Such stirring is required due to the local depletion of oxygen at the electrode membrane surface. Under the conditions employed in this study, vegetative cells of S. trochoidea appear not to have been adversely affected. This conclusion is based on the maintainance of linear rates of oxygen consumption or production by such cells over periods of many hours. The coherence of the P vs. I curve for S. trochoidea vegetative cells (Fig. 2), obtained over 4 hr, further supports the conclusion that incubation in the oxygen electrode does not siginificantly disrupt these cells.

Oxygen consumption by the electrode itself can be significant, particularly when small sample volumes are employed, and rates of biological consumption or production are low. The electrode consumption rate was measured daily, before and after each experimental run, in the light and in the dark. Statistical analysis revealed that over the course of a 5 day experiment, these electrode consumption rates were not different from day to day, but that the average dark consumption rate was significantly greater than the consumption rate in the light. This difference was accounted for in the calculation of respiratory and photosynthetic rates presented in Chapter 3.

Light-triggered germination. The care I employed in excluding incidental light during germination experiments increased throughout the time I worked on the problem, until manipulation in complete darkness became the standard procedure. The extreme sensitivity of S. trochoidea cysts to low photon fluences (Chapt. 2) justifies this

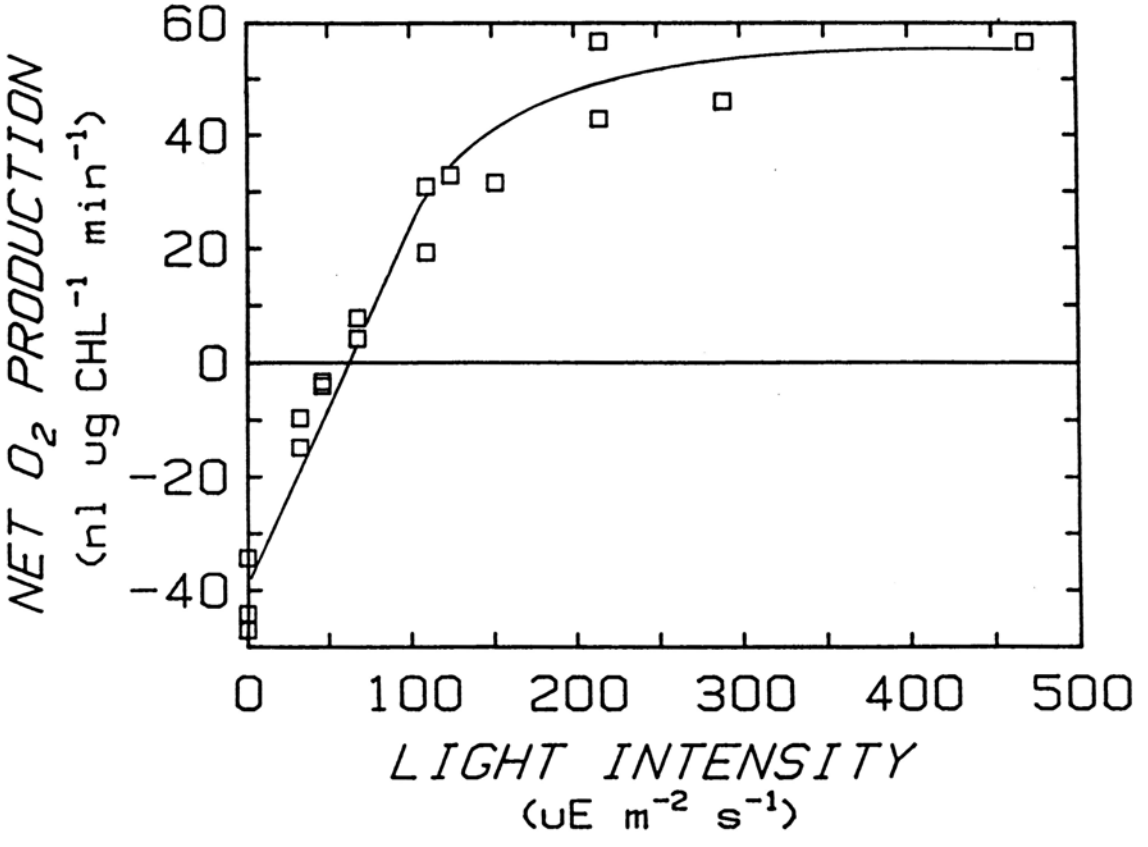

Fig. 2. Net oxygen production by vegetative S. trochoidea cells as a function of light intensity. Data normalized to Chlorophyll-a. Each point represents a single rate estimate from a 15 min exposure at the given light intensity.

care. Further characterization of the phenomenon may reveal that a red or far-red safe-light is indeed "safe," but for the time being complete darkness is recommended.

All cysts used in any particular light-exposure experiment were sampled from a single original susupension. For most experiments, light treatments were administered to triplicate 1 ml aliquots of cysts in 12x75 mm borosilicate tubes. After such treatment, these tubes were stored in light-tight boxes in a temperature controlled incubator. At weekly intervals, one tube from each treatment was removed, preserved, and assayed for germination in the usual manner (Chapt. 1). In this way differences in germination rate, as well as in ultimate germination frequency, could be detected. The light effects observed in the present study involved germination frequency exclusively; no significant additional germination was observed for any treatment after the first week of incubation. The three tubes which were sampled over time were therefore treated as replicates for each treatment.

Light treatments were administered in the specially constructed light-tight cardboard box illustrated in Fig. 3. To insure against extraneous temperature effects, cyst suspensions were held in an ice-water bath during light exposures. Dark-control cysts were held in the same bath for a comparable length of time. Light was provided by a 150 watt incandescent flood lamp. For "white" light fluence experiments, light intensity was adjusted exclusively with neutral density filters. In the case of wavelength-response experiments, the intensity was also adjusted by varying the supply voltage. This allowed finer control of exposure intensity, and was permissible

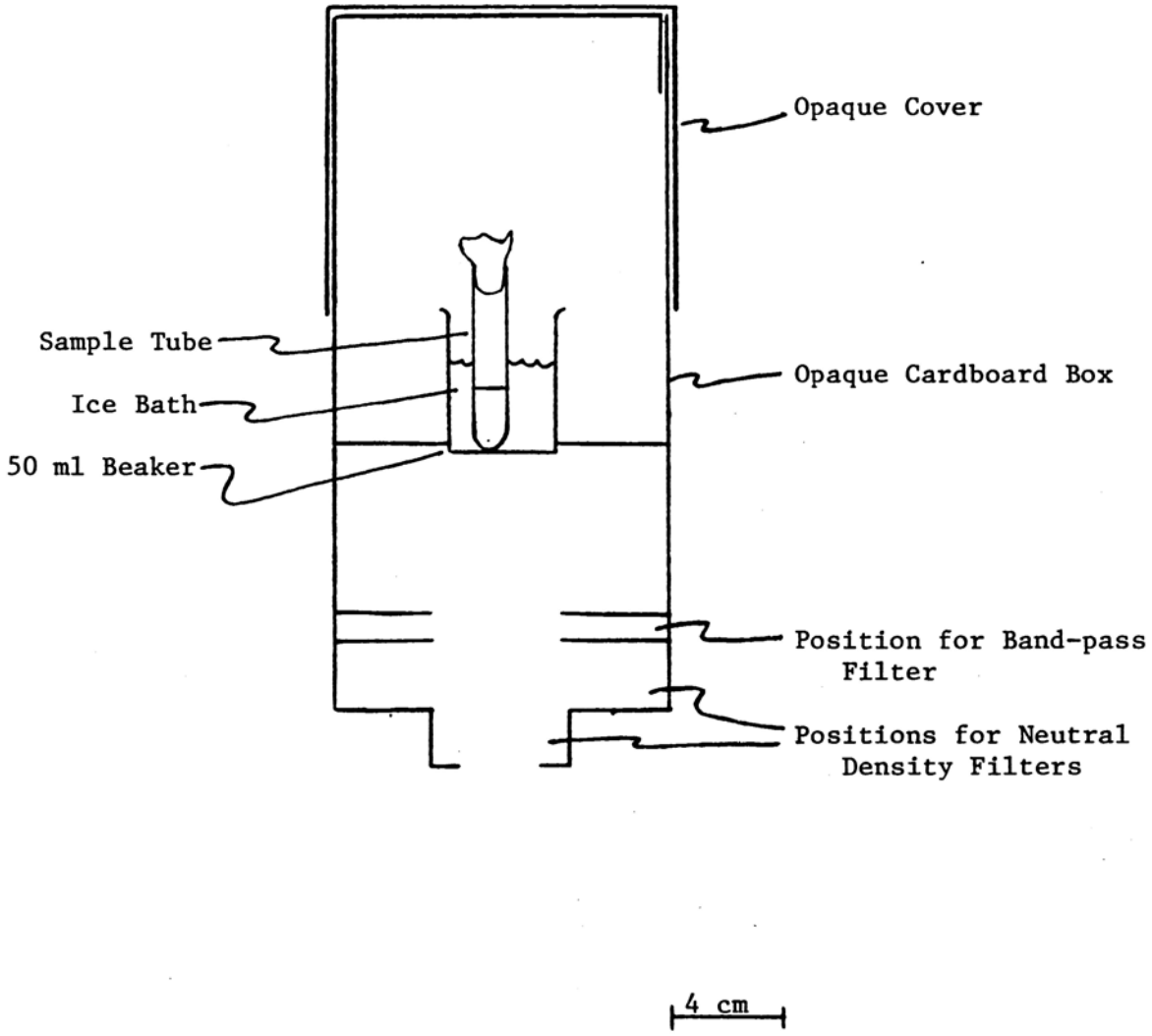

Fig. 3. Diagram of the box used to administer light treatments for photomorphogenic studies. Illumination is from the bottom; filter positions are accessed through slots in the front of the box; the sample bath is accessed from the top. Scale is approximate.

because the employment of 40 nm band pass filters made any resultant spectral changes in the source irrelevant. Exposure time was controlled by a darkroom timer.

The photon fluence rate for each particular combination of neutral density filters, band pass filters, and lamp voltage was measured with a scalar irradiance meter (Biospherical Instruments), except for the 700 and 750 nm band treatments, which were measured with a 2π radiometer (International Light) calibrated at lower wavelengths against the scalar meter. For very low light intensities, the actual irradiance was calculated from these measurements and the known optical density of the neutral density filters.

The band pass filters employed were 1 inch diameter interference filters (Ditric Optics) mounted in cardboard and carefully shielded to avoid stray light. These filters have a half-power band width of 40 nm, and are blocked to better than 0.1% transmission outside of the band, between low UV and 1000 nm.

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